The Journal of Infectious Diseases

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THE VIRULENCE ENHANCING ACTION OF MUCINS: A SURVEY OF HUMAN MUCINS AND MUCOSAL EXTRACTS FOR VIRULENCE ENHANCING ACTIVITY

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To invade epithelial surfaces, pathogenic bacteria must first fall on the mucin covering the mucosa and produce primary foci of infection in the presence of this mucin. The role of the mucin in this process is of importance in the study of all naturally occurring infections which take place on or across mucous membranes.

The classical function of mucin is that of lubrication and mechanical protection of epithelial surfaces, and it is reasonable to assume that this function can also be extended to antibacterial action. There is some experimental evidence for this view. On the other hand, the well established virulence enhancing power of hog gastric mucin for many pathogenic organisms in an artificial intraperitoneal infection of mice (Olitzi, 1948) is somewhat at variance with such an antibacterial function. It is of interest, therefore, to find out whether this virulence enhancement is confined to hog gastric mucin or is a general property of other mucins.

The present paper describes the collection of mucins and the preparation of mucosal extracts from various human sources and the results of assays for virulence enhancing action of these and a few other preparations. A biological assay for virulence enhancing activity involving an artificial intraperitoneal infection of mice with *Bacterium typhosum* (*Salmonella typhi*) in presence of mucin and a comparison with a "standard mucin" preparation have been described by Smith (1950a) and have been used in this work.

Only a few isolated observations for virulence enhancing activity of mucins other than hog gastric mucin have been recorded in the literature. Human salivary mucin (Anderson and Oag, 1939), (Tunnicliffe, 1940), (Sindbjerg-Hansen, 1943), human gastric mucin (Sindbjerg-Hansen, 1943), hog intestinal mucin (Levaditi and Vaisman, 1937) and guinea pig intestinal mucin (Cantacuzène and Marie, 1919) have been examined, and, with the exception of one sample of human salivary mucin (Sindbjerg-Hansen, 1943), found to be active.

EXPERIMENTAL AND RESULTS

Methods of extraction

Two methods were used: first a simple aqueous extraction, and second a method based on that used for extraction of hog gastric mucin (Smith, 1950b) involving peptic autolysis, an ethanol precipitation, and defatting and drying with ether. Two separately collected samples of each mucin or tissue were each extracted by these two methods.

Solid tissue.—The minced tissue was halved by weight. To one portion approximately twice the volume of distilled water was added, the mixture was shaken and allowed to stand at 0 C overnight. After centrifugation the tissue was re-extracted three times with the same volume of water. The combined extracts were strained through two thicknesses of butter muslin and freeze-dried. The

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"peptic extract" was prepared from the second portion by adding an equal volume of water containing 0.4 ml concentrated hydrochloric acid, 0.2 ml toluene and 1.0 mg of crystalline pepsin per 10 ml, and left at 37 C. On the second day the pH was adjusted to 2.5 to 3.0 and maintained at this figure for 5 days, 0.4 mg crystalline pepsin for each 10 ml of liquor being added on the third day. On the fifth day practically all the tissue had gone into solution; the liquor was strained through butter muslin, cooled, and 5 volumes of cold industrial ethanol were added. After standing overnight the precipitate was centrifuged off, washed once with industrial ethanol and twice with ether, and dried in a vacuum desiccator over phosphorus oxide.

Liquid mucins.—After collection liquid mucins were strained through two thicknesses of butter muslin and freeze-dried. The dried solid constituted the "aqueous extract." For the preparation of the "peptic extract" the solid was made into a 10% solution in water containing concentrated hydrochloric acid, toluene and crystalline pepsin, in half the concentrations described above. The rest of the process was exactly as described for the extraction of solid tissues.

Collection of specimens and yields of extracts obtained from them

I am indebted to Dr. P. E. Hughesdon, of the Department of Morbid Anatomy, University College Hospital Medical School, London, to Dr. H. W. H. Kennard, of the Pathology Department, Salisbury Infirmary, to Dr. J. E. Lovelock, of the Harvard Cold Research Unit, Salisbury and to Brigadier H. T. Findlay and my other colleagues at the Microbiological Research Department, Porton, for their help in collection of specimens. Each sample used for extraction was obtained by bulking specimens from more than one individual. Details of the samples used and the yields from both methods of extraction are summarized in table 1.

TABLE 1.—*Yields of aqueous and peptic extracts obtained from various sources.*

Source	Sample and quantity of mucin or tissue	Aqueous extract yield in g	Peptic extract yield (as % from aqueous extract; in g from one half of the original sample)
Human saliva	1. 1.5 L	14.0	31%
	2. 1.0 L	6.5	27%
Human nasal mucus	1.	12.6	58%
	2.	8.0	22%
Human bronchiectic fluid	1. 30 ml	2.0	
Human pseudomucinous ovarian cyst fluid	1. 400 ml (4 cysts)	15.0	34%
	2. 350 ml (5 cysts)	24.0	29%
Human stomach	1. 2 stomachs	10.0	5.0 g
	2. 5 stomachs	12.0	7.9 g
Human small intestine	1. Approx. 6 ft	8.0	4.5 g
	2. Approx. 20 ft	19.8	4.9 g
Human large intestine	1. Approx. 6 ft	7.2	5.0 g
	2. Approx. 12 ft	9.0	7.2 g
Human umbilical cord	1. 30 cords	8.0	6.0 g
	2. 30 cords	6.9	4.5 g
Hog gastric mucus	1. 500 ml	15.0	6.5 g
	2. 500 ml	17.0	5.1 g

Human saliva was collected from 20 to 30 individuals.
Human nasal mucus was obtained from the handkerchieves of the human subjects at the Harvard Common Cold Research Unit by extracting 3 times with water.
Sufficient bronchiectic fluid for one test only was obtained by bulking the fluid from three patients.
Human stomachs and small and large intestines were obtained from post mortem; excess fat and some of the thicker muscle were removed. It was impossible to separate the mucous membrane completely from external muscle before mincing.
The peptic extract from umbilical cords was precipitated from ethanol as a jelly; this was freeze-dried, since it proved impossible to dry it with alcohol and ether.
The thick hog gastric mucus was removed from the stomach linings of recently killed pigs.

The virulence enhancing activity of the various samples

The technique of the biological assay used for samples in the fractionation of hog gastric mucin and described by Smith (1950a) was used. The mucin preparation was made up in aqueous solution and after heating to 60 C for 45 minutes to kill vegetative pathogens, it was cooled, mixed with a dose of *Bact. typhosum* and injected intraperitoneally into mice. Comparison was made by probit analysis of the resulting death rate with that produced by a "standard" mucin in the same test. The strength of the unknown mucin in terms of the "standard" preparation was quoted in "Wilson units." 5000 *Bact. typhosum* organisms were mixed with the mucin preparations. A death rate of 20 to 80% in a batch of 20 mice indicated an increase in virulence of 1000

times since the dose of *Bact. typhosum* to give the same death rate in the same test when injected in tryptic meat broth is 5×10^6 organisms.

The results of the assays on all the above samples are given in table 2.

DISCUSSION

The results show clearly that the power of enhancing the virulence of *Bact. typhosum* is shared by all the preparations tested. It is probable that the virulence of other pathogens or potential pathogens would also be enhanced just as it is by hog gastric mucin (Olitzi, 1948), since the mucin most probably acts on the defense mechanisms of the host, not on the inherent virulence of the different bacterial species.

It is interesting to note that the activities of two totally different samples of mucin from the same anatomical source have about the same value. The greater activity of the liquid mucins over extracts from mucosal tissue is to be expected, since the latter are of necessity contaminated with extraneous material, especially the aqueous extracts. The extraction method which in-

TABLE 2.—*Results of the biological assay of the various samples for virulence enhancing activity.*

Source	Sample	Conc. (% w/v) of the mucin preparation 0.45 ml of which increases the virulence of *Bact. typhosum* 1000 times*	Activity of samples in "Wilson units" by direct comparison with the "standard mucin." Fiducial limits for $P = 0.95$ in parentheses
Human saliva	1. (a) Aqueous	3.5	0.29 (0.23–0.37)
	(b) Peptic	1.75	0.99 (0.67–1.41)
	2. (a) Aqueous	3.5	0.36 (0.30–0.43)
	(b) Peptic	1.25	1.10 (0.76–1.58)
Human nasal mucus	1. (a) Aqueous	3	0.46 (0.31–0.68)
	(b) Peptic	3	0.47 (0.34–0.66)
	2. (a) Aqueous	6	0.19 (0.09–0.32)
	(b) Peptic	2	0.69 (0.35–1.3)
Human bronchiectic fluid	1. (a) Aqueous	4	0.40 (0.20–0.60)
Human pseudomucinous ovarian cyst fluid	1. (a) Aqueous	10	0.10 (0.08–0.13)
	(b) Peptic	10	0.15 (0.12–0.20)
	2. (a) Aqueous	12	0.07 (0.02–0.12)
	(b) Peptic	10	0.21 (0.12–0.53)
Human stomach	1. (a) Aqueous	10	0.10 (0.07–0.13)
	(b) Peptic	3	0.36 (0.28–0.45)
	2. (a) Aqueous	10	0.14 (0.12–0.17)
	(b) Peptic	5	0.33 (0.24–0.49)
Human small intestine	1. (a) Aqueous	5	0.30 (0.25–0.37)
	(b) Peptic	5	0.25 (0.20–0.32)
	2. (a) Aqueous	10	0.14 (0.12–0.17)
	(b) Peptic	3.5	0.47 (0.39–0.57)
Human large intestine	1. (a) Aqueous	10	0.11 (0.09–0.14)
	(b) Peptic	6	0.31 (0.24–0.40)
	2. (a) Aqueous	10	0.11 (0.06–0.15)
	(b) Peptic	5	0.25 (0.16–0.34)
Human umbilical cord	1. (a) Aqueous	4	0.31 (0.25–0.39)
	(b) Peptic	10	0.20 (0.15–0.27)
	2. (a) Aqueous	5	0.28 (0.23–0.33)
	(b) Peptic	8	0.16 (0.14–0.20)
Hog gastric mucus	1. (a) Aqueous	2.5	0.56 (0.45–0.69)
	(b) Peptic	2	0.70 (0.56–0.89)
	2. (a) Aqueous	2.5	0.62 (0.51–0.75)
	(b) Peptic	2	0.91 (0.76–1.1)

Full details of the biological assay of virulence enhancing activity and the meaning of "Wilson units" are given by Smith (1950a).

Preliminary experiments were carried out to find the concentration of sample needed to give a death rate of 20 to 80% in a batch of 20 mice. In the final comparison with the "standard mucin," mucin blanks (20 mice) with no organisms ruled out toxicity of mucin samples at the particular concentration used. These results and the normal results obtained for the "standard mucin" death rates, organism control death rates and bacterial counts are not included in the table.

* Each mouse receives 0.45 ml of the mucin preparation together with 0.05 ml of tryptic meat broth containing 5000 *Bact. typhosum;* this mixture produces the same death rate (20 to 80% in a batch of 20 mice in 5 days) in the same test as 5×10^6 *Bact. typhosum* in 0.5 ml of tryptic meat broth.

volved hydrolysis with pepsin gave more active samples than the simple aqueous preparation, with the outstanding exception of human umbilical cord extract. The very high viscosity of the aqueous extract, compared with that of the peptic extract in this case, is probably the reason for this result (Smith, 1950b).

The preparations tested include two which are not derived from mucous membranes as such, namely, those from pseudomucinous ovarian cysts and human umbilical cord. It is true that the mucin from pseudomucinous ovarian cysts does, to some extent, resemble those from the mucosal surfaces in chemical constituents, e.g., in containing blood group substances (A and O), (King and Morgan, 1944; Morgan and Waddell, 1945), but the extract from human umbilical cord is quite different. It contains a very high proportion of hyaluronic acid. The activity of the latter preparation is, therefore, interesting.

Apart from the results of trauma, practically all pathogenic infection takes place on and across mucous membranes. It is surprising, therefore, that so little research has been carried out on the functions in this attack, of mucins which cover these surfaces. It may well be that the mucins have no marked effect on the course of the infection; but this has yet to be proved or disproved.

Extension of the classical function of lubrication and mechanical protection of epithelial surfaces to antibacterial protection is readily assumed, and there is some evidence for it. Goldsworthy and Florey (1930) studied the rate of disappearance of *Micrococcus lysodeikticus* and *Chromobacterium prodigiosum* from a colonic fistula of a cat. Their general conclusions favored the existence of some unspecified antibacterial mechanism resident in the intact mucosa. In this they agreed with the work of Eiskamp and Park (1921), and of Arnold (1929), who reviewed earlier work. Goldsworthy and Florey (1930) obtained discordant results in a search for definite antibacterial properties, due to the presence of lysozyme, in the alimentary canal mucus of cat, dog, rabbit and guinea pig. They suggested that any protective action which is common to mucus of all species against the entry of bacteria from the gut into the tissues must be sought rather in the mechanical properties of the mucus than in its ability to destroy bacteria. The thick mucus prevents the approach of the bacteria to the mucous surface, and the continual outpouring of mucin would tend to carry the bacteria away. Burnett and Stone (1947) have suggested that the mucinase of *Vibrio cholerae* is a possible factor in its pathogenicity, due to the reaction with mucin and the destruction of the mechanical properties of it. It is worth noting here that these same mechanical properties could prevent the host's defenses from attacking the bacteria.

The results above suggest another role for mucins, but it is impossible to infer from them that mucin definitely aids pathogens in their attack on mucosal surfaces. The activity in the mouse test might be ascribed to the production of an "artifact" during the process of extraction, but we believe this improbable in view of the very mild conditions used in the aqueous extraction, and the relatively robust properties of the virulence enhancing fractions of hog gastric mucin (Smith, 1950b). The salient point is that the mouse test, involving intraperitoneal injection of the organism with mucin, is artificial, and in no way resembles natural infection of mucosae. It is true that Hill, Huffer and Nell (1945) have found that *Neisseria gonorrhoeae* survive far longer on the mouse

vaginal surface when suspended in hog gastric mucin, than when suspended in saline, and the use of hog gastric mucin for the production of pneumonias by intratracheal instillation is a well established procedure (Olitzi, 1948). However, larger scale experiments on more natural infection of mucosal surfaces, by pathogens suspended in the mucin from the appropriate mucosa, have never been carried out. In view of the results recorded above, it seems clear that research in this field would be profitable in the sense that information might accrue relating to the mode of naturally occurring infection.

The widespread occurrence of the virulence enhancing factor in body fluids recalls that of the "virus receptor substance" (Stone, 1949; Friedewald, Miller and Whatley, 1947; Francis and Minuse, 1949; Svedmyr, 1949). Dr. E. Weston-Hirst of I.C.I. Ltd. Blackley, England, has very kindly examined our virulence enhancing factor of hog gastric mucin (Smith, 1950b) for inhibition of agglutination of red blood corpuscles by influenza virus A and B (living and heated). No such inhibition occurs, and any connection with the "virus receptor substance" would seem, therefore, to be ruled out.

SUMMARY

1. Preparations from human saliva, stomach, small intestine, large intestine, nasal mucus, pseudomucinous ovarian cyst fluid, umbilical cord, bronchiectic fluid and hog gastric mucus have been tested for activity in enhancing the virulence of *Bacterium typhosum*.

2. Without exception, all preparations proved to be active.

3. The confusing issue which thus arises of the role of mucins in the attack on epithelial surfaces by pathogens is discussed.

4. The virulence enhancing factor of hog gastric mucin has no connection with the "virus receptor substance."

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IN VITRO CULTIVATION OF THE MEXICAN STRAIN (VALLÉE "A" TYPE) OF FOOT-AND-MOUTH DISEASE VIRUS

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The large scale production of an effective vaccine against foot-and-mouth disease, commonly referred to as aphthous fever and at times "fiebre aftosa," has been generally accomplished by growing the virus in the tongue epithelium of live cattle. This method was used for making vaccine for field use in the campaign for control of foot-and-mouth disease in Mexico. Approximately 500 cattle were processed daily to obtain vesiculated tongue epithelium for use as a source of virus for vaccine. These animals were obtained in areas free of foot-and-mouth disease and shipped several hundred miles to the vaccine production unit. This was an extremely expensive and difficult undertaking when compared with methods used for making other types of vaccines.

During recent years the cultivation of viruses in vitro has offered a new field for an economical and efficient source of virus for production of certain vaccines. Attempts to cultivate the virus of foot-and-mouth disease in vitro have been made with varying degrees of success (Hecke, 1930, 1931, 1932; Maitland and Maitland, 1931; Frenkel, 1937, 1947, 1950). The successful growth of European strains as well as the Mexican strain (Vallée "A" type) was reported by Frenkel and Dunne (1949) as a result of work conducted at the State Veterinary Research Institute, Amsterdam, The Netherlands.

The objective of the work included in this report was to develop techniques for the in vitro cultivation of the Mexican strain (Vallée "A" type) of foot-and-mouth disease virus, with the ultimate aim of providing an economical source of virus for vaccine production.

Tissue culture studies

Animals used.—Approximately 520 cattle, of varying ages, were used in the tissue culture studies for virus titration, cross immunity and vaccination immunity tests. These animals were classed as susceptibles, since they were procured in Northern Mexico in areas free of foot-and-mouth disease.

Source of virus.—All in vitro virus cultivation passages were initiated with a Mexican strain (MP-VI) of the Vallée "A" type. This MP strain (Mexican Puebla) was identified by Galloway (1949) as Vallée "A" type, as a result of animal inoculation and complement fixation tests. The virus seed material, for experimental use and vaccine production, is identified by the date of its harvest. The following sources of the virus were used in initiating separate culture passages: (a) MP-VI (Sept. 29, 1949), (b) MP-VI (Nov. 4, 1949) and (c) MP-VI (Feb. 28, 1950). A filtrate of each culture was then used as the seed virus for inoculation of subsequent passages in each series.

Basic culture medium.—The ingredients of Baker's medium as given by Parker (1938) were modified for use as basic mediums in this tissue culture work. This medium had been used by Frenkel and Dunne (1949) for in vitro cultivation of foot-and-mouth disease virus. The basic

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† E. A. Eichhorn, VMD, and F. Camargo N., DVM, were joint Chiefs, Vaccine Production and Investigation Unit, the Mexican-United States Commission for the Eradication of Foot-and-Mouth Disease.

stock medium was prepared with the following ingredients per liter:

Sodium biphosphate..	0.0517	g
Sodium chloride.....	7.20	"
Potassium chloride...	0.18	"
Magnesium chloride..	0.09	"
Ascorbic Acid.....	0.0025	"
Phenol Red.....	0.0125	"
Calcium chloride.....	0.18	"
Sodium bicarbonate..	1.0	"
Dextrose.....	1.0	"
Peptone.....	6.750	"
Hemin.....	0.000036	"
Thyroxin.....	0.000009	"
Glutathione.....	0.1	"
Cystein hydrochloric acid.....	0.125	"
Vitamin A| Haliveroil Vitamin D/	0.4	ml
Isobuffer.....	32.	"
Dihydrostreptomycin base (sulfate).....	0.3	g
Penicillin G (Potassium).....	200,000.	units
Insulin.....	0.9	"
Water, Triple Distilled q.s.a.d.....	1,000.	ml

Preparation of tissues.—Frenkel et al (1949) showed that bovine tongue tissues could be shipped great distances for use in virus tissue cultures. This allowed the use of tissues obtained from animals in areas free of foot-and-mouth disease. In our work upper layer of tongue epithelium (stratum spinosum) and lower layer (stratum germinativum), obtained from cattle in Fort Worth, Texas, were shipped via air freight to Mexico City. These tissues were stripped from the tongue as soon as possible after slaughter of the animal. The tongues were prepared by washing thoroughly with soap and water, and then they were swabbed with 70% alcohol. The upper and lower layers of the epithelium were removed and rinsed in Tyrode solution. All tissues were shipped to Mexico as described below. Upon arrival the tissues were stored at approximately 5 C overnight in a serum-Tyrode solution. The tissues were then minced and washed three times in Tyrode solution. The buffer solutions for the third washing and for storing tissues in the refrigerator contained 200,000 units per L of penicillin G (potassium) and 0.3 g per L of dihydrostreptomycin base (sulfate). Before use in cultures, tissues during the third washing were allowed to remain one hour in the antibiotic buffer solution.

Initially, tissues from Fort Worth, Texas were shipped in Tyrode solution containing streptomycin (0.5 g/L) and penicillin (100,000 units/L). Later 20% bovine serum was added to the Tyrode solution. Approximately 10 g of the lower epithelial layer per 40 cc of serum-Tyrode solution and 50 g of the upper epithelial layer per 100 cc of the solution were the tissue-medium relationships used for shipment. After one to two days en route, the pH of the tissue medium in some instances dropped from approximately 7.4 to 6.6 or lower. For this reason, it was requested that 180 cc of serum-Tyrode solution be used for each 15 g of lower layer. When 50 g of upper layer were shipped in 180 cc, this amount of medium was inadequate for maintenance of optimal conditions. It was then requested that approximately 25 g of upper layer be shipped per 180 cc of medium. Even under the last conditions given, when tissues were delayed en route for more than two days, the pH of the medium dropped below 6.8. A pH of 6.8 was arbitrarily taken as the minimal requirement for tissue maintenance en route. Small Mason Vac-Seal bottles were found most suitable for shipment of tissues.

Harvest of culture virus.—In harvesting culture virus from routine experimental passages, the culture flasks were removed from the incubator, the supernate was separated from the tissue, and the latter was ground to a creamy consistency in a chilled mortar with the aid of quartz sand. The supernate and ground tissue then were mixed and centrifuged at 2500 r.p.m. for 15 minutes. The resulting supernate was filtered through a Seitz filter under 8 lbs pressure. The filtrate was then placed in sterile ampoules, sealed, frozen and stored as seed virus for inoculation of subsequent passages. Mortars and pestles were used to grind the epithelium for virus harvest in all of the work reported herein. However, since bovine tongue epithelium is difficult to grind in a mortar because of its resiliency, the high speed homogenizer, described by McCarty (1949) was tried in one test. It proved to be a simple method for breaking up tongue tissue and releasing virus. No marked differences in titer were observed in 5 titrations of material ground by the two methods.

Titration of virus.—The virus was titrated in cattle in a manner somewhat similar to that reported by Henderson (1949). The technique is one in which the tongue of an animal is used for inoculation of virus dilutions at multiple points. The method allows the use of 4 or 5 virus dilutions in one animal and obviates the use of large numbers of experimental animals. Five points were usually inoculated intradermally in each tongue with 1/10 cc amounts of each of 4 tenfold dilutions. Titration results were based upon positive or negative vesiculation at the inoculated points 28 hours after injection. The ID_{50} titer (50% infective dose) was calculated by the method of Reed and Muench (1938).

Virus diluted in beef heart broth adjusted to a pH range of 7.6 to 8.5 did not lose titer on short periods of storage. Broth adjusted to lower pH ranges showed loss of titer. For this reason beef heart broth adjusted to about pH 7.8 was used in preparing all serial dilutions of virus.

In titrating for virus content of infected tongue epithelium harvested from live cattle, the harvested material is weighed and considered as being pure virus for titration purposes. In the virus culture work reported herein, epithelium accounted for about 20% of the whole culture, while medium accounted for the remainder. In this work the whole culture was considered as pure virus in preparing titration dilutions.

Culture method no. 1.—This method of virus cultivation is essentially that used by Frenkel and Dunne (1949) in The Netherlands. This method proved successful in The Netherlands for growth of several strains of foot-and-mouth disease virus.

Erlenmeyer flasks of 500 cc capacity were used for cultures. The flasks were prepared with 125 cc of medium, from 18 to 24 g of minced epithelial tissues and 10 cc of the seed virus, all of which were added immediately prior to incubation. The medium and tissue covered the bottom of the flasks to a depth of approximately one inch. Upper and lower layers of the tongue epithelium were used in equal parts. Aeration of flasks was continuous with gas consisting of 95% oxygen with 5% carbon dioxide. This gas mixture was slowly bubbled through a capillary pipette into the medium for the full 24-hour incubation period. The rate of gas flow was adjusted to about 2 cc per minute. All cultures were incubated at 37 C. Cultures so managed caused a lowering of pH from 7.4 to about 6.8 at the end of the incubation period. Each of the following variations of the basic medium were used in virus cultivation trials with culture method no. 1:

1. Basic stock medium containing 20% ox serum ultrafiltrate.*

2. Basic stock medium containing 10% ox serum (inactivated at 56 C for one hour).

3. Basic stock medium containing the following added vitamins and amino acids:

Nicotinic acid	0.001	g/L
Pantothenic acid	0.0002	"
Riboflavin	0.00012	"
Thiamine chloride	0.00012	"
Pyridoxine hydrochloride	0.00006	"
Arginine, monohydrochloride	0.05	"
Lysine, monohydrochloride	0.18	"
(Peptone of basic stock medium was reduced to	3.00	")

4. Medium no. 3 above containing 10% ox serum (inactivated at 56 C for one hour).

5. Medium no. 3 above containing 20% ox serum ultrafiltrate.

Fourteen attempts were made to propagate the Mexican strain (Vallée "A" type) of virus in serial passage with culture method no. 1. Each of the 14 virus culture attempts was carried through 5 to 8 serial passages. (Seed virus for each successive passage was obtained from the previous passage.)

With one exception these passages proved negative on guinea pig or cattle inoculation on or before the 6th serial passage. The single exception was a serial passage that was virus positive upon guinea pig inoculation on the 7th and 8th passages and negative on the 9th passage.

The majority of cultures maintained in serial passage under this culture system were heavily contaminated. It was thought possible that the virus in these culture passages was destroyed because of contamination. Additional cultures were prepared; however, virus seed from only noncontaminated cultures were used in the serial passages. After three passages these cultures were virus nega-

* Obtained from Microbiological Associates, Post Office Box 5970, Bethesda 14, Maryland.

tive upon challenge inoculation of cattle.

Culture method no. 2.—This method of virus cultivation varied somewhat from culture method no. 1. Roux bottles (2″×4.5″×8″) with an offset neck were selected as culture flasks. The flasks were prepared with 100 cc of the medium described below, 4 to 25 g of minced tongue tissue and immediately prior to incubation were inoculated with 1 to 15 cc of the seed virus. After the flasks were laid on their sides the culture contents covered the bottom of the flask to a depth of about one-fourth inch. The surface area on the bottom of each flask was approximately 35 square inches. The shallow depth of medium and tissue was selected to allow maximum surface available for aeration. Pure oxygen or oxygen containing 5% carbon dioxide was mixed with equal parts of nitrogen. The atmosphere above the surface of the culture was replenished by allowing a slow and continuous flow of about 4 cc of gas per minute to enter the flask. All cultures were incubated at 37 C.

The tongue epithelium used in preparing cultures was cut by means of scissors into pieces approximately 2 to 3 mm square by 1 mm in thickness. This procedure causes considerable crushing of tissue cells. Smaller pieces of tissue would have been more desirable but were not used, since it was thought finer cutting with scissors would cause proportionately greater cell crushing.

Preliminary trials using culture method no. 2, were designed to obtain a tissue-medium relationship that would not allow utilization of all the available sugar in the basic medium containing 20% ox serum ultrafiltrate nor drop the pH from 7.4 to below 6.8 in a 24-hour incubation period. These trials gave evidence that up to 9 g of upper layer of tongue epithelium and 9 g of lower layer per 100 cc of nutrient medium would allow an incubation period for at least 24 hours without lowering the pH below 7.0 nor the sugar level below 25 mg per cent. Sugar determinations were made with the test reagent "*Galatest.*"* The pH was judged during incubation by phenol red indicator. Upon virus harvest, the pH of the medium was always determined with a Beckman pH meter.

Several series of cultures were prepared with 100 cc of basic stock medium containing 20% ox serum ultrafiltrate. The cultures were prepared with 18 g of equal parts minced upper and lower layers of bovine tongue epithelium. From 10 to 15 cc of the seed virus was used for culture inoculation. The cultures were incubated 24 hours at 37 C.

One specimen of MP-VI virus (29 Sept. 1949) was carried through 40 serial passages. Thirty-three of these culture passages were titrated, in each instance, by inoculation of tenfold dilutions at multiple points into the tongue of one cow. The ID_{50} titers obtained varied from $10^{-2.0}$ to $10^{-6.5+}$. Using this method most cultures gave virus titers in a range of $10^{-5.0}$ to $10^{-6.5}$.

The first 9 of the 40 serial passages were made in cultures that were aerated with approximately 48.5% of oxygen, 48.5% of nitrogen and 3% of carbon dioxide. The remaining cultures were aerated with equal parts of oxygen and nitrogen only, in an effort to maintain cultures at a higher pH.

When the 40 serial passages were initiated, 5 other series of cultures were carried for approximately 12 passages under identical conditions. Two separate samples of MP-VI seed virus were used. Both seed viruses were propagated in serial passage with only one of the 6 series failing to grow virus; it became virus negative on the third passage.

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* The Denver Chemical Mfg. Co., Inc., New York, New York.

Another sample of MP-VI virus, not previously used in tissue culture was started in 4 separate culture series, and each series was carried through 8 to 9 passages. Titration of these and previous series indicate the successful serial propagation of MP-VI virus in vitro using culture method no. 2.

For routine cultures 10 to 15 cc of seed virus were used for culture inoculation, although it was believed that this amount of virus was more than necessary. For this reason, a study was undertaken in which cultures were inoculated with 1, 3, 6, 9, 12 and 15 cc of 21st passage tissue culture virus. Cultures inoculated with these amounts of virus were carried through 5 additional serial passages. These were prepared with 100 cc of basic stock medium containing 20% ox serum ultrafiltrate, and 18 g of minced epithelial tissues (equal parts upper and lower layers). Cattle titrations of the 6 different series indicated that cultures inoculated with as little as 1 cc of seed virus would consistently produce virus titers of above $10^{-5.0}$. These were equal to the titers observed with cultures inoculated with 15 cc of seed virus.

Experiments conducted by Frenkel and Dunne (1949) at the State Veterinary Research Institute, Amsterdam, The Netherlands, suggested that virus growth in cultures was enhanced when certain vitamins and amino acids were added to the culture medium. This indicated that medium variation studies should be made using culture method no. 2. The mediums selected for study were as follows:

1. Basic culture medium.
2. Basic culture medium containing:

Nicotinic acid	0.001	g/L
Pantothenic acid	0.0002	"
Riboflavin	0.00012	"
Thiamine chloride	0.00012	"
Pyridoxine hydrochloride	0.00006	"
Arginine, monohydrochloride	0.05	"
Lysine, monohydrochloride	0.18	"
Peptone of basic stock medium was reduced to	3.00	"

3. Medium no. 2 above plus 20% ox serum ultrafiltrate.
4. Medium no. 2 above plus 40% ox serum ultrafiltrate.
5. Basic culture medium containing 20% ox serum ultrafiltrate.
6. Basic culture medium containing 40% ox serum ultrafiltrate.

Results of the medium studies are recorded in table 1. Basic stock medium alone failed to show virus growth on serial passage. Basic stock medium with added 20% ox serum ultrafiltrate appeared to contain the essential ingredients for growth of virus. The use of 40% ox serum ultrafiltrate showed no advantage over cultures prepared with 20% ox serum ultrafiltrate. The addition of vitamins and amino acids did not enhance the production of virus.

The results of titration of virus from cultures where the amount of tissue was varied and where only upper or lower layer of tongue epithelium was used are shown in table 2. Examination of these results revealed that cultures prepared with as little as 4 g of mixed upper and lower layer of tongue epithelium will produce virus in high titer throughout 5 serial passages. These determinations were made by injection of tenfold dilutions of virus at multiple points into the tongue of one cow. The titers were generally higher with 4 g cultures than for any series where a greater amount of tissue was used. It is possible these results were influenced by the effect of pH upon the virus content of cultures. In the course of this work it has been observed that cultures with a pH of above 7.0 contain more virus than those with a pH of below 7.0. Following incubation,

TABLE 1.—*Titration results (ID_{50}, 0.1 ml/point) of in vitro cultivated virus in cultures prepared with variations of medium components.**

Virus serial passage no.†	No variation	Vitamins +amino acids	Vitamins amino acids +20% ox SUF‡	Vitamins amino acids +40% ox SUF	20% ox SUF	40% ox SUF
13	$10^{6.6}$	$10^{6.7}$	$10^{4.2}$	$10^{4.1}$	$10^{4.8}$	$10^{6.3}$
14	$10^{6.8}$	$10^{6.6}$	$10^{6.0}$	$10^{6.5}$	$10^{6.7}$	$10^{6.2}$
15	$10^{6.0}$	$10^{6.6}$	$10^{6.2}$	$10^{4.0}$	$10^{4.8}$	$10^{6.0}$
16	$10^{6.0}$	$10^{6.9}$	$10^{4.5}$	$10^{4.0}$	$10^{4.4}$	$10^{6.6}$
27			$10^{6.0}$		$10^{6.5}$	
28			$10^{6.6}$		$10^{6.0}$	
29			$10^{4.0}$			
30			$10^{6.5}$		$10^{6.4}$	
31			$10^{6.2}$		$10^{6.4}$	
32			$10^{6.0}$		$10^{6.4}$	

* 100 cc of medium used for culture.
† Two series of culture passages are recorded. Seed virus for inoculation of the initial culture of each series was obtained from routine virus passages.
‡ Ox serum ultrafiltrate obtained from Microbiological Associates, Inc., Flemington, N. J.

cultures with more than 18 g of tissue gave pH readings of about 7.0, while those with less than 18 g of tissue gave readings up to 7.4.

Table 2 also gives a comparison of results of titrations of cultures prepared with upper versus lower layers of epithelium. There was some indication that cultures prepared with the lower layer only gave higher titers than those prepared with the upper layer of epithelium. Cultures prepared with the lower layer alone gave titers as high as has been generally observed where both upper and lower layers were mixed in equal parts. Due to the manner in which upper layers of tongue epithelium was stripped from the tongue, it was probable that some lower layer of epithelium was attached.

Several sets of cultures were prepared with 18 g of equal parts upper and lower layer of tongue tissue and inoculated with MP-VI virus for a comparison on virus yield in cultures incubated 24 hours with those incubated 44 hours. These were carried through 5 to 8 serial passages. Results of titrations of these cultures showed slightly higher titer for those incubated 24 hours than for those incubated 44 hours. The pH of 24-hour cultures was usually higher than in those incubated 44 hours. If a lower pH of cultures adversely affects the titer of virus, then this would explain why cultures incubated 24 hours might show more virus on titration than those incubated 44 hours. Cultures with smaller amounts of tissue maintain a more favorable pH for longer incubation periods. It is possible that the above results would reverse if 4 g of tissue were used in cultures incubated for the 24-hour and 44-hour periods.

TABLE 2.—*Titration results (ID_{50}, 0.1 ml/point) of in vitro cultivated virus,* using culture method no. 2,† in which the amount and type of tissue were varied.*

Virus serial passage no.	Equal parts of mixed upper and lower layers of epithelial tissue				Upper layer only	Lower layer only
	4g	11g	18g	25g	18g	18g
33	$10^{6.3+}$‡	$10^{4.8}$	$10^{4.0}$	$10^{4.6}$		
34	$10^{6.4}$	$10^{6.3+}$	$10^{6.3}$	$10^{6.7}$		
35	$10^{6.4}$	$10^{4.8}$	$10^{6.2}$	$10^{6.7}$		
36	$10^{6.2}$	$10^{6.3}$	$10^{6.0}$	$10^{6.0}$	$10^{6.2}$	$10^{6.2}$
37	$10^{6.0}$	$10^{4.5}$	$10^{4.2}$	$10^{6.4}$	$10^{6.5}$	$10^{6.2}$
38			$10^{6.0}$		$10^{6.0}$	$10^{6.2}$
39			$10^{6.0}$		$10^{4.0}$	$10^{6.0}$
40			$10^{4.7}$		$10^{4.2}$	$10^{4.2}$

* Seed virus used was obtained from routine virus passages.
† 100 cc basic stock medium plus 20% ox serum ultrafiltrate was used per culture (24-hour incubation at 37 C).
‡ + indicates insufficient dilution titration.

Control of bacterial contamination.—During the early period of this work, approximately 25% of the cultures prepared for virus harvest showed turbidity indicating heavy contamination. At one time the percentage of contaminated cultures was as high as 80%. Examination of 118 contaminated cultures yielded the following organisms in the frequency indicated: *Pseudomonas jaegeri* 60, *Pseudomonas aeruginosa* 24, *Pseudomonas convex* 2, *Nocardia coeliaca* 22, *Alcaligenes fecalis* 8, *Alcaligenes bookeri* 1, *Bacillus firmis* 1.

Since most of the above organisms are susceptible to the amount of antibiotics used in the culture medium, all measures used to prevent contamination were reexamined. When it was found that 80% of the cultures were contaminated, it was determined that a different type of streptomycin had been used inadvertently. This streptomycin was a calcium chloride complex product and upon addition to the basic stock medium, it was observed that a precipitate formed on the bottom of the medium containers. When a dihydrostreptomycin base (sulfate) was employed no precipitate formed. Approximately 250 subsequent cultures were prepared using 200,000 units per L of penicillin G (potassium) and 0.3 g per L of dihydrostreptomycin base (sulfate). None showed visible contamination as indicated by turbidity.

Of the previously mentioned 250 cultures none showed visible contamination, although when 126 of these were examined bacteriologically in Brewer's fluid thioglycollate medium, 120 revealed the presence of viable organisms. The organisms isolated from these cultures occurred in the following frequency: *Bacillus sphaericus* 88, *Serratia indica* 15, a yeast 2, and 15 belonging to either the genera of *Achromobacter*, *Pseudomonas*, *Micrococcus* or *Alkaligenes*. It was found that the amount of antibiotics used inhibited growth of these organisms to a point where the cultures did not become turbid upon incubation.

The minimal amount of antibiotics required for use in fluids for tissue storage, for tissue washing, and in culture medium was not determined. It is believed that the amount required depends upon the sterile precautions employed in preparing the virus cultures. The nature of tissue culture work, especially when bovine tongue tissue is used, requires that extreme precautions be taken at each step to avoid excessive contamination. Addition of antibiotics to tissue fluids and mediums at the time of their use, rather than at the time of their preparation aided in reducing contamination.

Cultures were prepared with 200,000 units per L of penicillin G (potassium) and from none to 0.6 g per L of dihydrostreptomycin base (sulfate) per L of medium. Antibiotics used in these amounts apparently did not inhibit virus growth in serial passage.

While the effect of other organisms on virus cultures was not determined, those inoculated with *P. convex* became virus negative after two to three serial passages.

When 200,000 units per L of penicillin and 0.3 g per L of dihydrostreptomycin were used routinely, these levels controlled bacterial contamination and permitted high virus yield.

Antigenic character of the tissue culture cultivated virus.—Complement fixation and cross immunity tests for the identification of foot-and-mouth disease virus grown in vitro were made in an effort to determine whether antigenic changes could be detected in MP-VI virus propagated in tissue culture. The complement fixation tests were made by the Serological Section of the Mexico-United States Commission for the Control of Foot-and-Mouth Disease. The tech-

nique for this test is described by Camargo et al, 1950.

Initially, tissue culture virus, i.e., filtrate, whole culture, and culture sediment were submitted for complement fixation tests. All these materials failed to fix complement. Because of this, tissue culture virus was inoculated into the tongue epithelium of cattle and when their tongues vesiculated the epithelium was harvested. Antigens prepared from this material consistently gave positive complement fixation results. Complement fixation tests were made with approximately 30 antigens extracted from the vesiculated tongue tissue of cattle that had been inoculated with from the first, to the 30th passage of tissue culture propagated virus. They were tested against the immune serums of 4 strains of Vallée "A" Type virus, one strain of Vallée "O" Type virus and one strain of Vesicular Stomatitis virus. Results of these tests were reported by Téllez Girón and Levine (1950). The results did not indicate any serological change in the antigenic structure of tissue culture virus, when compared with original MP-VI seed virus that had been used for primary inoculation of tissue cultures.

Results of cross immunity tests are recorded in table 3. The criterion for immunity is whether or not immune cattle will develop generalized lesions following intradermal inoculation of virus into the tongue. Only virus strains of the Vallée "A" Type that have been isolated in Mexico were available for these tests. They include those designated MP-VI, Jesús María, and Ameca. The facilities available limited the number of animals that could be used in these tests to 46 cattle. This number was insufficient to determine strain differences but was considered ample to determine whether aberrant antigenic forms of the virus developed as a result of tissue culture passage.

The results shown in table 3 indicate that animals recovered from infection with tissue culture passage MP-VI virus possess immunity against challenge with tongue propagated MP-VI virus. The results indicate that such recovered animals possess immunity against the other available Vallée "A" type virus strains. The latter observation also was

TABLE 3.—*Results of cross immunity tests of Mexican strains of Vallée "A" type virus.*

Animals			Results of virus challenge*		
No. used	Susceptible or recovered from virus infection	Average days of recuperation	Virus strain	Percentage protection	
				Local	Generalization‡
1	Susceptible		MP-VI	0	0
3	MP-VI	108	a	0	100
1	Susceptible		Ameca	0	0
3	MP-VI	109	a	66	100
1	Susceptible		Jesús María	0	0
3	MP-VI	108	a	33	100
1	Susceptible		26 T.C.†	0	0
3	MP-VI	108	a	100	100
2	Susceptible		MP-VI	0	0
6	1 to 18 T.C.†	34	a	100	100
2	Susceptible		Ameca	0	0
6	1 to 18 T.C.	33	a	100	100
2	Susceptible		Jesús María	0	0
4	15 T.C.	33	a	100	100
2	Susceptible		26 T.C.	0	0
6	1 to 18 T.C.	33	a	100	100

* Animals challenged by inoculation of 0.05 cc of 1:100 virus suspension at 2 points intradermally on the tongue.
† T.C. = Tissue culture passage MP-VI virus. Numeral indicates passage number.
‡ Generalization refers to extension of the disease to the foot parts.

seen in animals immune to and cross challenged with the other virus strains.

Tissue culture vaccine studies

Preparation of the vaccine.—During the early period of the tissue culture work reported, there was speculation as to how much virus could be taken from each culture for vaccine production. There was a question as to how much virus was contained in (1) the tissue suspending culture medium, (2) the supernate of a ground culture, (3) the filtrate of the latter, and (4) fluids used to wash virus from the sediment of a ground and centrifuged culture. In order to investigate these problems, 5 titrations were made on each of 12 different cultures. The virus used in these cultures was of the 15th to 22nd tissue culture passage material. When these titrations were compared it was found that in the culture medium alone the virus ID_{50} titer was 0.8 of a log less than in the ground and centrifuged culture supernate. After the latter was filtered through a Seitz filter employing an EK pad, it was found that there was 0.6 of a log loss in virus titer. The centrifuged culture sediment was resuspended in virus-free basic stock medium in an amount equal to the original culture medium. This was considered a first washing of culture tissue. The centrifuged supernate of this material contained an amount of virus equal to that found in the supernate of the culture processed as usual. When this same sediment material was rewashed, there was a loss of 1.5 log in virus titer. Because of the above, cultures prepared for production of virus for experimental lots of vaccine were processed as usual and in addition the centrifuged tissue sediment was washed in virus-free basic stock fluid in a volume of one to one-half times the original culture medium. The supernate of the centrifuged tissue was pooled as virus for vaccine in both cases. With this procedure approximately 250 cc of virus were harvested from each culture.

All vaccines prepared from tissue culture virus were made by the method that was currently in use for making tongue tissue vaccine in Mexico. The details of the vaccine production procedures are included in the report "*Activities Carried Forth at the Vaccine Production Division*," Mexico-United States Commission for the Eradication of Foot-and-Mouth Disease, Mexico D.F. The vaccine is prepared by mixing a virus suspension derived from vesiculated tongue epithelium with an aluminum hydroxide colloid so that each 2 cc dose represents 0.11 g of epithelium.

The aluminum hydroxide colloid is made by adding an 8% solution of ammonium hydroxide to a 5% solution of aluminum sulfate until a pH of 7.4 is reached. The colloid is washed 4 or 5 times in centrifugal extractors until it contains less than 60 ppm of ammonium hydroxide and less than 30 ppm of ammonium sulfate.

It was found that the tissue culture virus and the aluminum hydroxide colloid had to be placed under a vacuum of at least 54 cm mercury for a minimum of 15 minutes to withdraw adsorbed gases. This procedure was omitted when Lots 1 and 2 of vaccine were prepared and considerable foaming resulted in the final mixtures. These vaccine lots were considered unsatisfactory for immunity studies.

The virus-hydroxide complex is thought to be absorbed more slowly and to provide antigenic stimulus over a longer period of time than a simple formalized virus. Schmidt (1940) showed the significance of such an adsorbate vaccine. Rosenbusch et al (1948) reported upon the successful use of an intradermal dose of 2 cc of an aluminum

hydroxide adsorbed foot-and-mouth disease vaccine. Aluminum hydroxide adsorbed vaccine has been extensively used in South America. Results of immunity tests of animals vaccinated in Mexico have shown this type of vaccine to be satisfactory.

Lot 3 of tissue culture vaccine was prepared with 1835 g of aluminum hydroxide colloid. This colloid was adjusted to pH 8.0 by adding a glycocoll buffer adjusted to pH 9.8. The colloid concentration was 2.75% calculated upon a basis of dry oxide content. To the colloid was added 2000 cc of 26th passage tissue culture virus that gave a titer of $10^{-5.4}$ upon titration in the tongues of 2 cattle. After the above mixture was agitated for 20 minutes, 4.3 cc of 37% formaldehyde solution diluted in 225 cc of distilled water were added. Agitation was continued for 15 minutes. The vaccine was then incubated 48 hours at 25 C. The vaccine was tested for bacterial sterility. Tests were made in cattle to determine that the virus component of the vaccine was inactivated and would not produce foot-and-mouth disease.

Vaccine immune studies.—A vaccine potency test of Lot 3 of tissue culture vaccine was made in conjunction with the Tacuba Vaccine Test Unit of the Vaccine Production Division. In this test, each of 20 cattle was inoculated intradermally with 2 cc of Lot 3 of tissue culture vaccine. In addition, 6 animals used on virus innocuous tests were included in the vaccine potency tests. Three of these animals were given 50 cc of vaccine subcutaneously and 3 were given 50 cc intramuscularly. The animals were challenged after 15 days to determine the protection induced by the vaccine. Of the 20 vaccine test animals 10 were exposed by contact with an infected donor animal. The remaining 10 vaccine test animals and the 6 innocuity animals were exposed by intradermal inoculation at two points on the tongue, of 0.05 cc of a 1:100 suspension of native MP-VI virus.

Three lots of tongue tissue vaccine were tested simultaneously with the tissue culture vaccine. On the basis of the test used, the tissue culture vaccine gave 23% less protection than that which was shown by the simultaneously tested tongue tissue vaccine. Six animals inoculated with 50 cc each of Lot 3 of vaccine were afforded complete protection against generalization of the disease upon intradermal challenge on the tongue with native MP-VI virus. These results indicated that 2 cc of tissue culture vaccine was an insufficient dose to provide the same degree of protection as was induced by the tongue tissue vaccine. The 50 cc dose of tissue culture vaccine gave 100% protection against generalization of the disease.

The procedure for preparation of Lot 4 of tissue culture vaccine was identical with that of Lot 3. The virus employed in production of this vaccine gave a titer of $10^{-4.8}$ upon titration in the tongues of two cattle. The titer of this virus was approximately 0.5 of a log lower than that used in vaccine Lot 3. Vaccine potency tests were made employing varied doses. Three groups of 20 animals each were inoculated with 2 cc of vaccine intradermally, 4 cc and 8 cc subcutaneously, respectively. In addition 6 animals were inoculated with 25 cc of vaccine; 3 animals subcutaneously and 3 intramuscularly. The 4 groups of animals were tested by virus challenge in the same manner as those vaccinated with Lot 3 vaccine. Results of these tests indicated that a 4 cc dose of Lot 4 of tissue culture vaccine was required to produce protection in cattle comparable to that afforded by 2 cc of a simultaneously tested tongue tissue vaccine.

In an endeavor to correlate the relative immunity induced by the tissue culture vaccine against that produced by the tongue tissue type on the basis of tissue content, total nitrogen determinations were run by the Kjeldahl-Cuning-Arnold method on Lots 3 and 4 of tissue culture vaccine and Lots 381 and 382 of the tongue tissue vaccine. Results indicated that the tissue culture vaccine contained approximately 40% less total nitrogen. These findings raised the question as to whether an excessive amount of formalin had been added to the tissue culture vaccine, since on the basis of titer, 1 cc of the tissue culture virus was considered the equivalent of 1 cc of the epithelial virus suspension, whereas, in reality the latter contained the greater amount of nitrogen.

COMMENTS

The technique employed with culture method no. 1 was similar to that used successfully in The Netherlands by Frenkel and Dunne (1949). This method did not prove successful in Mexico. MP-VI virus was, however, successfully cultivated by using culture method no. 2. One of the essential differences between these methods was the system of aeration of cultures. In culture method no. 2 the Roux flasks employed allowed greater medium surface available for aeration than was allowed by the Erlenmeyer flasks used for culture method no. 1. In addition the depth of the medium in the Roux flasks was only about one-fourth of an inch as compared with one inch in the Erlenmeyer flasks. It was not determined why culture method no. 1 was not successful in Mexico but was successful in The Netherlands. Consideration should be given to differences in altitude at the two places that may have had a bearing on the systems employed in aeration of cultures. The Palo Alto Laboratories in Mexico are at an altitude of approximately 8000 ft., while the laboratory in The Netherlands is approximately at sea level.

Mexican and United States authorities responsible for the program for eradication of foot-and-mouth disease in Mexico decided that all work with virus, i.e., virus production for vaccine and all virus research, would be discontinued when it had been determined that mass vaccination of Mexican livestock was no longer required. As a result of this decision tissue culture research with the virus of foot-and-mouth disease was discontinued before optimal methods for production of a tissue culture vaccine could be established. It is believed that the work reported herein at least provides a sound basis for further developments of an efficient and relatively inexpensive tissue culture vaccine for protection of livestock against foot-and-mouth disease.

SUMMARY

A method is described for the successful propagation of a Mexican strain (MP-VI) of the Vallée "A" type of foot-and-mouth disease virus. This virus was carried through 40 tissue culture serial passages. The cultures routinely showed virus titers, in susceptible cattle, in a range of $10^{-5.0}$ to $10^{-6.5}$. Eight additional culture series were carried successfully through 8 to 12 passages under identical conditions. A 10th culture series failed to grow virus beyond the 3rd serial passage.

The method that proved successful for the propagation of the virus in vitro employed Roux flasks as culture containers. To 100 cc of culture medium were added from 4 to 25 g of equal parts of upper and lower layers of minced bovine tongue epithelium. The flasks were inoculated with from 1 to 15 cc of seed virus and then laid on their sides

in an incubator maintained at 37 C for 24 hours. During this period the cultures were aerated by a slow flow of equal parts of oxygen and nitrogen that replenished the atmosphere above the surface of the culture.

Although 10 to 15 cc of seed virus were used for routine inoculation in culture passages, 1 cc of seed virus was later found to be sufficient.

Cultures prepared with 4 g of equal parts of upper and lower layers of bovine tongue epithelium showed virus titers as high as in cultures prepared in amounts up to 25 g.

When a modification of Baker's fluid was tried as the medium for propagation of the virus in tissue cultures the virus failed to grow on serial passage. When 20% ox serum ultrafiltrate was added to the modified medium it then contained the essential ingredients for virus growth. The addition of nicotinic acid, pantothenic acid, riboflavin, thiamine chloride, pyridoxine, arginine and lysine to the medium failed to enhance the growth of virus.

There was no evidence as determined by cross immunity and complement fixation tests that the serial propagation of the MP-VI strain of virus in tissue cultures changed its antigenic structure or produced aberrant forms.

Cattle recovered from induced infection with MP-VI virus, that had been serially passed through tissue cultures, were solidly immune when challenged with native MP-VI virus and the homologous tissue culture virus.

Virus propagated in tissue culture was used to produce an aluminum absorbate vaccine similar to that made with virus propagated in the tongues of live cattle. It was found on limited trials to produce satisfactory immunity in cattle exposed to the native MP-VI virus.

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THE EFFECT OF X-IRRADIATION ON RATS WITH AND WITHOUT *BARTONELLA MURIS*

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Studies carried out in the past several years provide ample evidence that infection and infectious factors play an important role in radiation disease. With certain infections the laboratory animal develops natural and/or acquired resistance. For example, *Bartonella* infections have been observed in many animal species and the specific role of the spleen in the maintenance of resistance to these infections has been pointed out.[1] A mass of evidence has accumulated which points to a definite lowering of natural resistance to spontaneous or induced infections and to acquired resistance to latent infections as a result of splenectomy. The drop in resistance applies to many species of animals but probably is not of a general character. Animals manifesting this lowering of resistance are referred to as infected or *Bartonella*-carrier animals, while those not developing such a reaction are regarded as infection-free or *Bartonella*-free, since they do not have the spontaneous or induced infections.[1] With the removal of the spleen there occurs, in the infected animal, a profound anemia, leukocytosis and frequently death.[1,2] Aureomycin, an antibiotic agent reported to benefit the rat subjected to whole body lethal radiation,[3] will prevent the development of the post splenectomy anemia; while rutin, a flavonoid agent without benefit to the irradiated rat,[4] will also reduce the post splenectomy anemia in the nonirradiated rat.[2] With the above implications in mind, studies were undertaken, and the data reported here serve to demonstrate a difference in mortality rates of infected and infection-free rats when exposed to total body lethal X-irradiation.

METHODS AND MATERIAL

The Sprague-Dawley and Slonaker-Addis strains of rats were selected and examined according to the schedule noted below. The Slonaker-Addis strain has been bred and studied for many years by other investigators in this laboratory. It is infected with *Bartonella muris*. This strain of rats was fed a stock diet from a common storage bin.* The Sprague-Dawley rat was bred and raised at Madison, Wisconsin, under very rigid techniques to prevent infections. It does not have the *B. muris* infestation. These rats were shipped to this laboratory at weanling age (about 21 days),

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* The feeding of this stock diet converts the *Bartonella*-free to a *Bartonella*-infected rat. It is presumed that the handling of the cages, the animals with *B. muris* and the stock diet by the same personnel without precautions is a medium for the transmission of the disease. This subject is under further study.

2. Rekers, P. E. and Marti, N. F. 1950, The Effect of Aureomycin and the Flavonoid Rutin on the Splenectomized Rat. Am. J. M. Sc. In press.
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when they were placed on a special diet, the USP XII Vitamin A Test Diet* with alpha and beta carotene† added. This diet was not contaminated with the *Bartonella*. All animals were housed in the same animal room, in small groups, in separate cages. No special precautions were adopted other than to insure feeding the designated diet to the designated animal.

The technique of single dose total body X-irradiation was given to all rats. A Picker X-ray machine of 220 KVP, 15 Ma, delivered 6 r per minute at a target skin resistance of 45 inches. A parabolic copper filter with a half value layer of 1 mm of copper was used which gave uniform irradiation over the horizontal plane at the anticipated level of the skin, 45 inches from the target of the X-ray tube. The dosage delivered to the dorsum of the skin was determined from direct measurement in air with Victoreen ionization chambers at trial runs. Rats were irradiated in a flat cylindrical wheel with 20 equal sectors, the control and experimental rats being placed in alternate compartments. The wheel was rotated 180° periodically during irradiation. The rats had some freedom of motion in a horizontal plane throughout the irradiation period. Rats weighing 150 g received 700 r, while those weighing 200 g received 750 r.

Mortality data were computed from a 30-day period of observation following irradiation.

Schedule of rats undergoing irradiation.—Group 1. Adult male Sprague-Dawley rats, born in Wisconsin, shipped to this Institute at weanling age and fed the U.S.P. XII vitamin. A test diet with carotene added. An infection-free rat.

Group 2. Adult male Sprague-Dawley rats born at this Institute and fed the U.S.P. XII vitamin A test diet with carotene added. The parents, however, received the stock diet during the breeding and gestation periods. An infected rat #1.

Group 3. Adult male Sprague-Dawley rats, born in Wisconsin and treated as in group 1 except that 10 days before irradiation the stock diet was substituted and continued thereafter. An infected rat #2.

Group 4. Same as group 1. Diet supplemented with aureomycin,‡ 20 mg per day per rat beginning one day before irradiation. An infection-free rat.

Group 5. Same as group 1. Diet supplemented with aureomycin,† 20 mg per day per rat beginning one day before irradiation and rutin§ 100 mg per day per rat beginning 10 days before irradiation. An infection-free rat.

Group 6. Adult male Slonaker-Addis rats on stock diet. An infected rat #3.

The infected state due to *Bartonella* infection was determined by the demonstration of anemia, leukocytosis and *Bartonella* bodies in the blood smear following splenectomy. Rats were selected at random from the above categories for splenectomy but not irradiated or included in the mortality computations.

Sprague-Dawley rats were converted to the infected state by feeding a stock diet, which probably was a means of inducing the *Bartonella* infestation. The second generation Sprague-Dawley rats harbored the *Bartonella* infection and may have acquired it from the parents who were fed the stock diet or by feeding on the stock diet just before weaning. The transmission of the *Bartonella* infection is under further study.

RESULTS

Data from these experiments are recorded in table 1 and figures 1 and 2. Infection-free rats showed a mortality of 91% and 92%, with death occurring between the 5th and 21st days after irradiation. Similar mortality curves were obtained with the 150 g rat exposed to 700 r and the 200 g rat exposed to 750 r single dose, total body X-irradiation. Infection-free animals receiving aureo-

TABLE 1.—*Effect of X-irradiation on infected and infection-free rats.*

Type of rat		No. of rats	Supplement	Mortality %	X-Ray r
Infection-free	S-D*	25	Aureomycin and rutin	92	750
Infection-free	S-D	10		90	750
Infected #1	S-D	18		50	750
Infection-free	S-D	21		91	700
Infection-free	S-D	20	Aureomycin	95	700
Infected #1	S-D	18		42	700
Infected #2	S-D	10	Stock Diet	50	700
Infected #3	S-A†	19	Stock diet	47	700

* S-D Sprague-Dawley Rat
† S-A Slonaker-Addis Rat

* U.S.P. XII diet obtained from General Biochemical Inc., Laboratory Park, Chagrin Falls, O.

† Carotene was generously supplied through the courtesy of Dr. H. Bennett, 6256 Cherry Avenue, Long Beach, Calif.

‡ Aureomycin was generously supplied through the courtesy of Dr. J. Ruegsegger, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

§ Rutin was generously supplied through the courtesy of Mr. Giles St. Clair, S. B. Penick Co., Church Street, New York 7, N. Y.

mycin with or without rutin presented similar mortality rates and curves. In contrast to the above findings all groups of rats in the infected state manifested a delay in mortality as well as a decreased mortality rate to the 50% range. This observation applied to the Slonaker-Addis strain of albino rat as well as the Sprague-Dawley animal.

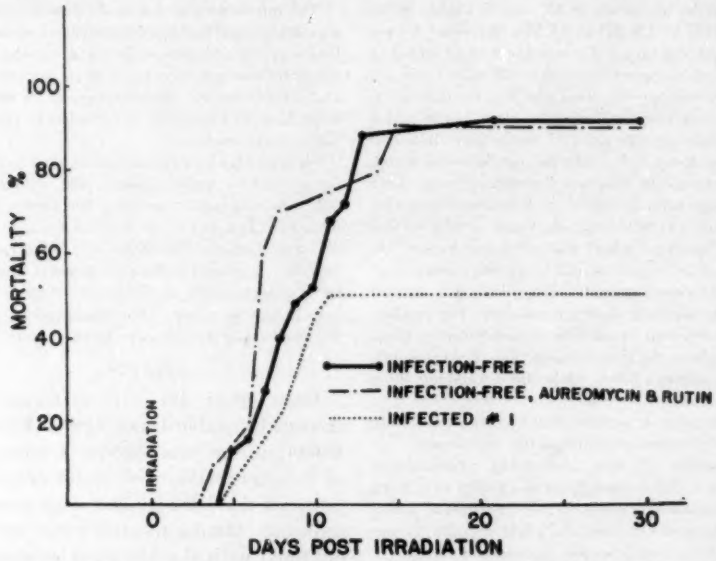

FIG. 1.—Mortality of infected and infection-free rats following X-irradiation with 750 r.

Careful clinical and postmortem examinations did not educe significant differences between the infected and infection-free animals. Weight loss was greater in the infection-free group, but these data were sharply influenced by the steeper slope of the mortality curve as well as the higher mortality rate. Watery diarrhea was not a conspicuous finding—not even in those rats fed the more coarse, the stock diet. The hemorrhagic tendency was more common in the dissected animal, especially in the alimentary tract where the stomach and upper intestines were filled with blood, while the pulmonary system and testes appeared less involved. A mass kill phenomenon was not demonstrable, particularly in those animals receiving aureomycin therapy. Organ weight data were difficult to correlate and interpret in the face of the marked total body weight loss and hemorrhage into tissues. Hypoplastic changes in the hemopoietic tissues, as anticipated, were present in all animals.

DISCUSSION

The infection acquired spontaneously or induced in the rats under study was with the *B. muris*. It is possible that other common laboratory infections occurred as well, but their identity was not established by the tests employed. The *Bartonella* infection serves to demonstrate a common laboratory infection, the pathology of which has been well documented.[1] In as much as the spleen has been described as playing an

essential part in the maintenance of resistance to this infection,[1] it is well to inquire further into the role the spleen may play in the process of infection and that of irradiation disease. The importance of the spleen in the mechanism of natural resistance to certain acute and chronic bacterial and protozoan diseases, and of acquired resistance to latent infections is well established.[1] The importance of the spleen in antibody formation is strongly suggested but not unequivocally established.[1,7] Likewise, the nonirradiated spleen in the otherwise irradiated animal offers protection from the deleterious effects of irradiation.[5] This protective effect is believed to be due to ectopic erythrocytopoiesis in the shielded spleen.[5] When an antibiotic agent such as aureomycin is given to the irradiated rat, there is almost complete alleviation of the diarrheal state and prolongation of the survival period.[3] When aureomycin is administered to the nonirradiated, *Bartonella* infected, splenectomized rat, there is a block to the expected anemia and leukocytosis.[2] In the study with aureomycin on the irradiated rat, it is probable that the antibiotic agent exerted its effect on the spontaneous and/or acquired infection, as well as by suppressing certain organisms in the intestinal tract. The inability of aureomycin to influence the course of irradiation disease in the noninfected rat, reported herein, lends further credence to the view that the drug exerts its action on an existing infection.

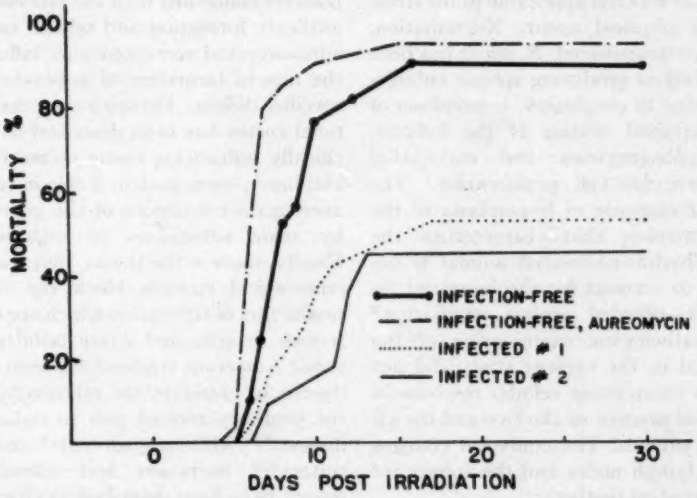

FIG. 2.—Mortality of infected and infection-free rats following X-irradiation with 700 r.

With the infected animal manifesting a prolongation of the survival period and a reduction in mortality, it is to be inferred, then, that the infection has set up a defense mechanism within the host that has not only provided resistance to the infection but an increased tolerance to the stress of irradiation. Whereas the resistance conferred for the infection may be specific, it is not to be construed

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that any such specificity has accrued for one or more of the many factors that result from irradiation. This apparent defense mechanism is developed rapidly. The infection-free rat that is converted to the infected state by feeding an apparently contaminated diet 10 days before irradiation also shows the reduced mortality curve. The question whether or not the *Bartonella* infection has, in effect, produced an antigen-antibody response, a humoral effect or a cellular response which is applicable to the stress of the physical agent, X-irradiation, must go unanswered. *B. muris* has been described as producing splenic enlargement due to congestion, hyperplasia of the germinal centers of the follicles, erythrophagocytosis and endothelial and reticular cell proliferation.[1] The cellular response of hyperplasia of the bone marrow that characterizes the phenylhydrazine-treated animal is believed to account for the increased resistance afforded against irradiation.[6] Histopathological examination of the material in the present study did not show a conspicuous cellular response in the bone marrow or the livers of the infected animals. The equivocal changes in the lymph nodes and the spleen are being studied further.

Morphological and physiological studies have established the role of the adrenal glands in processes concerned with both natural and acquired resistance contributing to the defense of the organism, particularly against intoxications, infectious diseases and environmental stresses.[7] The physiological relationships between the adrenal cortex and the adrenal medulla are intimate. The adrenal cortex has been described as influencing the defense mechanism in several ways:[7] 1) the level of secretory activity of the cortex may be influenced by the antigen in a nonspecific manner by affecting pituitary-adrenocortical secretions, 2) increased quantities of circulating adrenocortical steroids may have an effect on the cells of the reticuloendothelial system which are, in turn, possibly concerned with the processes of antibody formation and release and 3) adrenocortical secretions may influence the rate of formation of antibodies by specific tissues. Furthermore, the adrenal cortex has been described as specifically influencing tissue permeability and may play a profound role in resistance against invasions of the organism by toxic substances or organisms.[8] Finally, there is the theory that the adrenocortical steroids block the tissue production of substances which are deleterious to cells and cause cellular injury.[7] Numerous studies have been conducted to elucidate the relationship of the pituitary-adrenal axis to radiation disease.[9,10] Although adrenal[4,9] and pituitary[4,10] hormones and adrenalectomy[11] have been described as affecting morbidity and mortality in a variety of

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species subjected to irradiation, further observations are necessary to clarify this provocative subject.

SUMMARY

Rats free of a common laboratory infection, such as *Bartonella muris*, show a mortality rate of 90% when exposed to single dose, total body X-irradiation. When these rats become infected with *B. muris* for 10 days or longer and are then irradiated, there is not only prolongation of the survival period but a reduction in the mortality rate to 50%. It is proposed that the induction of the infection has not only conferred a resistance to the infection but also an increased tolerance to the physical agent, X-irradiation. The mechanism for this effect is not elucidated by these studies.

BIOLOGICAL STUDIES ON *ENTAMOEBA HISTOLYTICA*

III. INDUCED ENCYSTATION IN SEVERAL MEDIUMS, INCLUDING AN ACCOUNT OF A NEW PROCEDURE

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The biology of *Entamoeba histolytica* still presents several fundamental enigmas. Investigation of nutritional requirements has been seriously handicapped by inability to cultivate trophozoites free of other organisms. Understanding of the other important stage of the life cycle, the cyst, is hampered by ignorance of the causes of encystation in vivo and in vitro, as well as by the often cited practical difficulty of obtaining large numbers of mature cysts. Cysts have found use in many experiments dealing with epidemiological factors, and owing to their relatively greater resistance, have been used more frequently than trophozoites to initiate cultures with changed bacterial associates.

Dobell and Laidlaw (1926) were apparently the first to produce encystation in cultures, although with "uncertain results" (p. 302); their method involved withholding an essential component (rice starch) from their diphasic RES medium (egg slants with serum-Ringer overlay) for one or two subcultures, and then providing it. In a more thorough study Cleveland and Sanders (1930) obtained cysts in two different diphasic mediums (liver-infusion-agar slants and Loeffler-serum slants, each with serum-saline overlay and added rice starch). Stone's (1935) encystment medium consisted of buffered Locke's inorganic salt solution with added whole wheat flour. Chang (1942, 1946) has made the most complete study of factors concerned with encystation, especially the role of pH and oxidation-reduction potentials. Using both monophasic (buffered liver infusion) and diphasic (Cleveland's liver-infusion-agar slants with buffered serum-saline overlay) encystment mediums, he concluded that the onset of encystation was usually correlated to a sharp, unfavorable rise of potential following a period of accelerated trophic growth. In Chang's opinion a good encystment medium is one containing a rich supply of reducing substances favoring accelerated multiplication. The pH values for good encystation covered a lower range (6.4 to 6.8) in his later work than in the earlier one (6.8 to 7.1). Chang (1943, 1950) also has performed a valuable service by collating older data on factors influencing longevity of cysts and by contributing new data. Kessel et al (1944) compared yields in several encystment mediums as a function of pooling starch-depleted inoculums from Dobell's egg medium versus Cleveland's liver medium. They concluded that best yields were obtained in modified Cleveland's liver-infusion-agar slants with serum-saline overlay, following one rice-free transfer to Cleveland's liver medium. Zuckerman and Meleney (1945) reported large yields of cysts under reduced atmospheric pressure (40 mm mercury) in flasks containing an all-fluid medium: cholesterol, proteose peptone, cerophyl, serum and Ringer's solution.

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The general conclusion of Cleveland and Sanders still seems valid today; in their opinion three essential conditions for encystation are: (a) accelerated growth of amoebae for 24 hours or more; (b) addition of rice starch to rice-free cultures; and (c) the presence of certain (undetermined) bacteria in the medium. To these might be added adjustment of pH and a highly reduced medium. These conditions permit such a wide latitude in devising suitable encystment mediums that it is not surprising that the above methods often have not proved successful in other laboratories. Evaluation of the various procedures would require information about the "encystment response" (percentage of cysts formed), total numbers of cysts harvested, and the proportion of mature (quadrinucleate) cysts. It is unfortunate that data concerning one or more of these points have usually been omitted.

In this laboratory we have required large numbers of cysts for initiating monobacterial cultures in egg yolk infusion, in which population cycles of amoebae have been traced (Balamuth and Howard, 1946; Balamuth and Wieboldt, 1951). In seeking a reliable procedure to induce encystation we were led, like Kessel, to compare several available mediums. In the elimination series which ensued a new medium gave best results. Since several other laboratories now have used our method successfully,* we have been encouraged to describe it together with an account of the evidence for its selection.

EXPERIMENTAL

Preliminary tests

The NRS strain of *E. histolytica* was used in these early tests, since it had been found that the associated bacterial flora readily permitted encystation (Balamuth and Wieboldt, 1951). The previously described mediums of Dobell, Stone, Chang (diphasic), and Kessel were compared, using 16 ml amounts (solid and liquid components in a ratio of 1:3) in 150×18 mm Pyrex test tubes. To these were added a few local mediums based upon the following principle: if an encystment response follows only a period of accelerated growth, eggyolk infusion might prove useful, since it is easy to dilute or concentrate the over-all nutrients while maintaining essentially isotonic conditions. Trial showed that the amoebae could tolerate half-strength infusion (prepared by diluting the stock infusion with Ringer, 1:1), and that double-strength infusion (prepared with twice the regular concentration of eggyolk) promoted greater than normal growth. Thus by cultivating the trophozoites rice-free at depressed nutrient levels in dilute infusion, attenuated amoebae could be pooled for inoculation into a highly reducing medium containing double-strength infusion. One kind of local medium contained an overlay of double-strength eggyolk infusion over a buffered liver-agar slant (see next section for details); the sources of liver were Wilson liver concentrate powder 1:20 and Lilly liver extract powder 343. Monophasic variations were prepared by adding 0.5% concentrations of several different liver extracts (Wilson liver fractions B, E, S, and the above whole-liver concentrate†) to double-strength eggyolk infusion. All mediums were inoculated with rice-depleted cultures pooled from Dobell's and Cleveland's mediums, and in some runs with the local mediums dilute eggyolk infusion was also tried. At least four runs lasting approximately 48 hours were conducted with each medium, and duplicate tubes usually were carried in eact test.

Qualitative observations showed that (a) the published mediums ranked in the order: Kessel, Chang, Dobell, and Stone, with the last two proving most erratic; (b) the diphasic local medium ranked first, while the best monophasic ranked about on a par with Chang's medium; (c) no difference could be detected between the Lilly and Wilson whole-liver extracts, but they were distinctly superior to the special fractions; (d) the inoculums pooled from Cleveland's medium gave higher yields than from Dobell's; (e) variations in pH were greatest in the unbuffered Dobell's medium, but the reaction was slightly alkaline (7.0 to 7.4) in all at 48 hours.

* Most recently Mr. M. C. McCowen of the Lilly Research Laboratories has reported our medium "very excellent for cyst production," obtaining average cyst yields of 65% to 75% (letter of Sept. 6, 1950).

† The various Wilson liver extracts were obtained through the courtesy of Dr. David Klein, Wilson Laboratories, Chicago, Ill.

In the course of these runs it became apparent that several variables were influencing the encystment response, among which were the necessity of exceeding a minimal inoculum and the condition of the amoebae in the inoculum. The latter evades any quantitative evaluation and yet is probably the most important single factor involved in insuring accelerated growth culminating in encystment.

It was decided to make a more critical comparison of Kessel's and our diphasic mediums, with special reference to the percentage of cysts formed, the relation of inoculum to yield, and the relative advantages of egg and liver pre-encystment mediums. Details on preparation of the new medium will be given first.

New encystment medium

Base.—For preparation of 100 ml use 2 g of Wilson liver concentrate powder 1:20; add to 80 ml distilled water, bring to boil, and filter; add 6.4 ml of 0.25 molar $Na_2PO_4 \cdot 12H_2O$ and 7.6 ml of 1.0 molar potassium phosphate buffer (ratio of 4.7 parts K_2HPO_4 to 0.3 parts KH_2PO_4). With distilled water in volumetric flask bring above mixture to 100.0 ml. Transfer to beaker and add 3.0 g Bactoagar. Heat gently until agar dissolves. Autoclave 20 minutes at 15 lb pressure. The pH should be about 7.2.

Overlay.—To double-strength eggyolk infusion (twice usual concentration of eggyolk) and normal horse serum, in ratio of 10:1, rice starch is added last, amount being regulated according to requirements of bacterial flora and amoebic inoculum.

Inoculating encystment tubes.—In the following experiments 200×25 mm Pyrex tubes were used to receive 10 ml base (slanted and solidified), 30 ml double-strength eggyolk infusion, 3 ml horse serum (in some series), and rice starch. The pre-encystment amoebae were carried for several transfers in tubes of half-strength eggyolk infusion, subculturing each 48 hours and gradually depleting the cultures of rice until no free rice starch remained. The sediment from several tubes was pooled, adjusted to a standard volume for counting on haemocytometers,* and inoculated into the encystment medium. The maximum encystment response in our experiments usually has occurred at 48 to 72 hours, although samples must be removed and stained with iodine periodically to determine the optimal endpoint.

Harvesting cysts.—Most of the overlay was carefully drawn off without disturbing the sediment. The agar slant was removed from the tube by means of a needle bent into a hook; thrusting the slant up and down will free most of the sediment. The remaining fluid and sediment were transferred to a centrifuge tube, adjusted to a known volume, and the total population counted. The contents then were spun at 2000 r.p.m. (C. S. & E. centrifuge, type 118-F, radius 7.5 inches) for 2 minutes, after which the supernatant was replaced with sterile distilled water, the contents mixed, and the rinsing was repeated two more times. The tube was then stored at 4 C for at least one day to kill trophozoites, while the mature cysts remained viable for several weeks or months. The final count of cysts was made in distilled water after adjusting to a known volume.

TABLE 1.—*Comparative encystation of the NRS strain of* E. histolytica *in Kessel's medium and a new medium.*

Encystment medium	Pre-encystment medium	Total yields ($\times 10^{-3}$)			Yields of cysts ($\times 10^{-3}$)			% Encystation (av)
		Min	Max	Av	Min	Max	Av	
New medium (no serum)	Dilute eggyolk infusion	2170	5746	3372	939	3352	1700	50.4
	Cleveland's	1692	2811	2270	238	818	499	22.0
New medium (with serum)	Dilute eggyolk infusion	1214	8133	3930	936	5641	2287	58.2
	Cleveland's	1169	6403	3079	58	305	199	6.5
Kessel's	Dilute eggyolk infusion	1086	8661	4220	68	896	310	7.3
	Cleveland's	1548	4120	3039	6	24	12	0.4
Base: New Overlay: Kessel	Dilute eggyolk infusion	2752	6457	5330	1006	2097	1567	29.4

Comparison of Kessel's medium with new medium

Several bases for comparing the two encystment mediums have been grouped in tables 1 to 3. Table 1 shows that the largest encystment responses occurred

* Each recorded value for a given inoculum was the average of all squares on six separate haemocytometers.

in the new medium containing horse serum, and that inoculums derived from dilute eggyolk infusion (8 experiments with duplicate tubes in each case) gave consistently higher yields of cysts than those derived from Cleveland's medium (3 experiments with duplicate tubes in each case). The distinction between enhancement of growth and encystation is evidenced by the fact that Kessel's medium yielded the largest number of trophozoites. The inoculum in all cases exceeded 20,000 amoebae. Variability was observed between the duplicate tubes in some experiments, but in those cases it was usual to find that the tube (table 1). A distinct improvement was noted in encystment response. In our experiments, therefore, the solid phase of Cleveland's medium (used in modified form in Chang's and Kessel's encystment mediums) disclosed distinct limitations.

Several of the comparative runs were designed to permit a more critical evaluation. As shown in table 2 this was accomplished by using identical inoculums in four series, and the results generally conformed to the above pattern. One interesting point concerns the relation of size of inoculum to total yield. It is apparent that, irrespective of the encyst-

TABLE 2.—*Comparative encystation of* E. histolytica (*NRS strain*) *in different mediums in the presence of identical inoculums.*

Exp	Encystment medium	Pre-encystment medium	Inoculum ($\times 10^{-3}$)	Yields of cysts ($\times 10^{-6}$)	% Encystation
1	New (no serum)	Dilute eggyolk infusion	31.2	625.0	55.3
	Kessel		31.2	2.8	0.1
2	New (no serum)	"	41.8	1658.5	64.4
	New (with serum)		41.8	1515.0	43.7
3	New (no serum)	"	50.6	1801.0	51.2
	Kessel		50.6	449.0	10.8
4	New (with serum)	Cleveland's	231.8	304.5	4.8
	Kessel	"	231.8	5.5	1.3

containing more trophozoites also had more cysts. In one run involving the new medium with horse serum, for example, duplicate inoculums of 60,000 trophozoites gave total yields of 2,710,000 and 4,175,000 amoebae, with corresponding cyst yields of 1,906,000 (70%) and 2,647,000 (63%). Pre-encystment cultures in Cleveland's medium were always more abundant than in dilute eggyolk infusion; indeed, it seems probable that the more successful use of the latter medium was correlated to the greater attenuation of growth therein, permitting a greater acceleration upon transfer to the encystment mediums. The relative efficacy of the solid slants in the two mediums was studied by adding Kessel's overlay to the new base in 4 experiments ment response, there was in these particular series a direct dependence of total yield upon the size of inoculum. (Total yield is readily calculated as the product of cyst yield times the reciprocal of percent encystation.)

The relation of inoculum to total yield also was studied by adjusting the size of inoculum over a threefold range in given experiments with the new encystment mediums (table 3). In all cases greater numbers of cysts were obtained with larger inoculums. A complicating factor was occasionally introduced through the correlation of a larger inoculum to a shorter and sharper encystment response. In experiment 3, for example, the larger inoculum yielded an earlier encystment response and

TABLE 3.—*Encystation of* E. histolytica *(NRS strain) in a new encystment medium as a function of size of inoculum.**

Exp	Encystment medium	Inoculum ($\times 10^{-3}$)	Yields of cysts ($\times 10^{-3}$)	% Encystation
1	New (no serum)	93.8 281.4	1320.0 3352.0	33.9 58.3
2	New (with serum)	31.6 94.8	3613.0 5641.0	85.1 69.4
3	New (with serum)	40.0 120.0	331.0 1553.0	13.0 73.5

* Amoebae for all experiments were pooled from dilute eggyolk infusion.

fewer total amoebae (2,113,000 versus 2,542,000 from the smaller inoculum). The onset of the encystation obviously terminates multiplication and thereby lessens the total yield of amoebae.

The question naturally arose, whether other strains of *E. histolytica* would react like the NRS strain used in the above experiments. This was the same strain used by Kessel et al, although their particular substrain did not become available to us. Several other strains have been tested with the new encystment medium in this and other laboratories. With one exception encystation has occurred. The exception was a strain provided by Dr. John L. Bradin (then a graduate student at the Johns Hopkins University), which was designated as N. I. H. strain 103-t and had never been reported to encyst in the particular flora present. Abundant growth was readily obtained, but no cysts were ever recovered. Representative data are given in table 4 for the UC strain; this strain had been recovered originally from a dysenteric patient in 1939 by Dr. Enid de Rodaniche at the University of Chicago. The trends agreed with those in table 1, although Kessel's medium proved more favorable than with the NRS strain.

DISCUSSION

The practical control of encystation of *E. histolytica* is admittedly a difficult problem. In the present work we have been especially impressed by the importance of the intrinsic condition of the amoebae in determining the outcome of encystation. The advantage claimed for our procedure rests above all upon transferring attenuated amoebae from a diluted, less nutrient medium to a highly nutrient one containing a common factor of eggyolk. The ensuing accelerated multiplication of amoebae culminated regularly in encystment, in contrast to other procedures submitted to comparative tests. The data emphasize that for proper evaluation of encystment response records must include the percentage of cysts formed as well as the total number. Furthermore, since cysts undergo extensive development and only the mature, quadrinucleate

TABLE 4.—*Comparative encystation of the UC strain of* E. histolytica *in Kessel's medium and a new medium.*

Encystment medium	Pre-encystment medium	Total yields ($\times 10^{-3}$)			Yields of cysts ($\times 10^{-3}$)			% Encystation (av)
		Min	Max	Av	Min	Max	Av	
New medium (no serum)	Dilute eggyolk infusion	1350	2408	1696	447	932	700	41.0
	Cleveland's	1868	7311	3959	162	804	367	9.2
Kessel's	Dilute eggyolk infusion	1780	3674	2771	309	1338	646	23.3
	Cleveland's	4009	8550	6280	456	559	507	8.0

stage is viable (Chang, 1943), proper evaluation also requires information on this point. Limited assistance available in the present study prevented thorough analysis of relative yields of immature and mature cysts. However, actual excystation tests and D'Antoni's iodine stain were applied throughout all experiments as guides to formation of quadrinucleate cysts, and the termination of given runs was controlled by the appearance of this stage. Both Kessel's and the new medium yielded mature cysts, but our qualitative records indicate that the new medium delivered greater percentages of these, as well as less of vacuolated, nonviable cysts in general.

The role of the bacterial flora is probably crucial in encystation, although still unknown in its details. Chinn et al (1942) found that encystment was rare in monobacterial cultures of any kind studied by them. In mixtures of known bacteria *Clostridium perfringens* was the only type which consistently stimulated growth and encystation, while *Clostridium sporogenes* had the opposite effect of proving amoebacidal. This approach has not been pursued by others. In the present work involving the NRS strain of *E. histolytica* analysis of the associated flora had disclosed only three intestinal types: *Escherichia coli communior*, *Escherichia coli anaerogenes*, and a paracolon bacillus (Balamuth and Wieboldt, 1951), and yet encystation occurred regularly and in higher proportion than reported by Chinn et al, 1942. The flora of particular cultures can be readily modified at the present time in several ways: micro-isolation of sterile cysts (Chinn et al, 1942), mass treatment of cysts with bactericidal agents (Balamuth and Wieboldt, 1951), or selective inhibition of an existing flora by antibiotics while "swamping" most survivors by inoculating treated cultures into an actively growing flora already known to permit encystation (Chang, 1943). Either of the last two methods is readily available to any laboratory with limited mechanical facilities.

The oxidation-reduction potential of amoebic cultures is controlled chiefly by the bacteria present. Chang's (1946) data showed a frequent correlation between a sharp rise of potential and the onset of mass encystation, and this suggests one important way in which bacteria may influence encystation. Experiments still under way in this laboratory thus far have not confirmed this correlation, although our curves show the expected attainment of E_h values between -200 and -300 millivolts during the phase of active growth in the encystment tubes. One interesting factor which came to light during these experiments concerned the influence on encystation of bottom surface in culture vessels. Electrolytic beakers (70×45 mm) were used at first because of greater ease of manipulation but were abandoned upon discovering that encystation was not induced in these vessels, despite prolific trophic growth. The importance of this factor was clinched through introducing identical inoculums into tall tubes (200×25 mm) and electrolytic beakers, both containing 40 ml medium. Only in the former case did encystation occur. This illuminates once again the great complexity of the problem and calls for more critical study of the role of all causal factors at work.

SUMMARY

1. Several existing encystment mediums were compared, and ranked as follows in evoking an encystment response in the NRS strain of *Entamoeba histolytica:* Kessel, Chang, Dobell, and Stone.

2. A description is given of a new diphasic encystment medium, consisting

of a buffered liver-agar slant and an overlay containing double-strength egg-yolk infusion and horse serum (10:1), with added rice starch.

3. A critical quantitative comparison between Kessel's and the new medium disclosed the superiority of the new medium in inducing encystation. Measurements were made of inoculums, total yields of trophozoites, and of cysts. The bases of comparison included use of both fixed and varied inoculums, use of the same base with different overlays, and the pooling of inoculums from eggyolk infusion and Cleveland's medium.

4. The results were confirmed with the UC strain of *E. histolytica*.

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FURTHER OBSERVATIONS ON METABOLIC CHANGES IN ORAL LACTOBACILLI

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Fermentation changes in strains of oral lactobacilli have been described by Harrison[1] and further by Coolidge et al.[2] The fermentation change consists in an acquisition of the ability to ferment sorbitol and mannitol, usually accompanied by the production of acid, coagulation, and reduction in litmus milk. Occurring simultaneously are a change from the heterofermentative to the homofermentative type of metabolism and a loss in thiamin requirement for growth. Strains which have not undergone these changes are here termed "nonfermenters"; those which have changed are called "fermenters."

In the present paper it is shown that such changes occur, not only in cultures isolated and propagated in the usual manner, but also in cultures grown from single cells, and that the rate of change is not increased by growth in thiamin-free medium. Confirming earlier results, it is shown that some factor associated with the preparation of washed suspensions of the organisms increases the incidence of the change.

METHODS

Source of Cultures.—Four strains of oral lactobacilli were isolated from four children 14 years of age who were patients in the Zoller Memorial Dental Clinic. From each of these strains a single colony was isolated and a new culture grown from this colony. From each of these single-colony cultures a single cell was isolated and a new culture grown from this cell.

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Strain RH37E was isolated in 1942 from the saliva of an oral cavity containing carious teeth. The strain was then a nonfermenter. After six months in stock, the strain had changed to a fermenter. The single colony which initiated the single-colony and single-cell strains used in this study was isolated from a tube of the original strain which had been frozen in dry ice when the culture was a nonfermenter and which was still a nonfermenter after thawing. The single-colony and single-cell strains were both nonfermenters when isolated in 1946 and remained so until the changes occurred which are described in this paper.

Strain EH41D was isolated in 1942 from a dental plaque on a carious tooth. The strain was then a nonfermenter. After two years in stock, the strain had changed to a fermenter. The single colony which initiated the single-colony and single-cell strains used in this study was isolated from a tube of the original strain which had been frozen in dry ice when the culture was a nonfermenter and which was still a nonfermenter after thawing. The single-colony and single-cell strains were both nonfermenters when isolated in 1946 and 1947 and remained so until the changes described in this paper occurred.

Strain EH68H was isolated in 1944 from a dental plaque on a carious tooth. The strain was then a nonfermenter. After six months in stock, the strain had changed to a fermenter. After four more months, it had changed back to a nonfermenter and remained so. The single-colony strain used in this study was isolated after the original strain had reverted to the nonfermenting stage. The single-colony strain and the single-cell strain isolated from it were both nonfermenters when isolated in 1945 and remained so until the changes herein described occurred.

Strain EH78A was isolated in 1944 from a dental plaque on a carious tooth. The strain was then a nonfermenter. A tube of this culture which had been frozen in dry ice for five months proved to be a fermenter upon thawing. The single-colony strain used in this study was isolated in 1945 from this fermenting culture of the original strain and was itself a fermenter upon isolation. After six months in stock, this single-colony strain had become a nonfermenter and remained so until it changed as herein described. The single-cell strain

was isolated during the period when the single-colony strain was fermenting, but the single cell was a nonfermenter and so remained until the changes occurred as described.

Stock cultures of these four single-colony and four single-cell strains were kept in Jay's broth[3] or in tomato juice yeast extract (TJY) broth[4] and transferred every four to six weeks. All 8 cultures remained consistent nonfermenters for a period of from two to four years before these studies were initiated.

The technique of single-cell isolation used was a modification of that of Reyniers.[5] A microneedle made of tungsten wire, described by Trexler,[6] was attached to a micromanipulator. Under the microscope the needle was drawn through a freshly made streak of organisms on a solidified agar drop and out onto a fresh agar surface. The locations of organisms falling off the needle singly were marked. The colonies which grew up in these marked locations were thus known to have arisen from single cells.

Mediums.—The mediums used were tomato juice yeast extract (TJY) broth, and three semisynthetic mediums differing only in their thiamin concentration. The first of these mediums, *a*, is a modification of the synthetic medium of Henderson and Snell,[7] the modification being: (1) the replacement of the mixture of individual amino acids by casein hydrolysate and *l*-tryptophane to give 1 g of casein hydrolysate (General Biochemicals) nitrogen per liter and 320 mg of *l*-tryptophane per liter; (2) the elevation of the glucose concentration to 2%; (3) a thirtyfold elevation in the vitamin concentration. The second medium, *b*, is identical with the first, except that the thiamin concentration is only half that of the first. The third medium, *c*, is identical except that it contains no thiamin. Fermentation tests were made in the mediums used by Harrison,[8] the carbohydrates being used in 0.5% concentration.

Transfers, washings, and fermentation tests

Method 1.—Inoculations were made into test tubes of semisynthetic medium *a*. From 5 to 7 serial transfers in this medium were made over a period of from 10 days to two months, by which time the growth of each strain was well established. The cells in the last of these transfer tubes were removed by centrifugation, washed three times with sterile 0.85% sodium chloride solution, and suspended in a volume of the saline equal to the volume of medium in which they had been grown. From these suspensions 0.2 ml inoculations were made to tubes of semisynthetic mediums *a*, *b*, and *c*. These tubes were incubated at 37 C for 2 to 4 days. Cells from that medium giving good growth which contained the smallest amount of thiamin were washed and reinoculated into the semisynthetic mediums. This process of selecting the culture growing in the least amount of thiamin, washing it, and reinoculating it into semisynthetic mediums was repeated at intervals of usually 2 to 4 days over a period of 5 months before changes in fermentation were observed. Fairly frequently the medium containing no thiamin supported growth, and the culture from this medium was that used for the next washing. Except for occasional erratic results, growth was usually good in medium *b* as well as medium *a*, and transfers to medium *a* were, therefore, discontinued. When, occasionally, good growth did not appear in 2 to 4 days, the tubes were incubated further before washing; a few of the cultures selected for washing had been incubated 6 to 8 days, and a few at that time still showed rather poor growth. When no growth appeared in any of the tubes in a series, a tube with better growth which had been saved from a previous transfer, or a fresh subculture from such a tube, was used for washing. (In two cases the process was started over again from the stock culture.) From each tube which showed good growth inoculations were made directly into tubes of litmus milk and sorbitol and mannitol broths before washing. Each washed suspension was also tested for fermentation of milk, sorbitol, and mannitol.

Each of the four single-cell cultures was carried through this process until a fermentation change occurred. The single-colony cultures were not treated in this way.

Method 2.—Cultures in tubes of TJY broth, made freshly from stock cultures, were incubated for one to three days and then washed three times in sterile 0.85% saline solution as described above. Fermentation tests in milk, sorbitol, and mannitol were done on these cultures before washing and on the saline suspensions after washing.

Each of the single-cell cultures was treated from 4 to 8 times by this method, beginning each time with the stock culture; each of the single-colony cultures, from 2 to 7 times.

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Method 3.—During the period of time when the procedures used for methods 1 and 2 were being followed, the cultures were repeatedly transferred to TJY broth and to semi-synthetic medium without intervening washing of the cells. Fermentation of milk, sorbitol, and mannitol by the resulting subcultures was tested.

The total number of such transfers, with subsequent fermentation tests, of single-cell cultures was 56; that of single-colony cultures was 18.

RESULTS

Method 3.—Of the 56 transfers of single-cell cultures with no intervening washing, 27 were from semisynthetic medium to semisynthetic medium, 17 were from TJY broth to TJY broth, 9 were from TJY to semisynthetic, and 3 were from semisynthetic to TJY broth. The 18 transfers of single-colony cultures were all from TJY broth to TJY broth. No change of fermentation was found in any of these cultures which had not been washed.

Methods 1 and 2.—In single-cell cultures the number of washings of cultures grown in semisynthetic medium (method 1) was 146, and that of cultures grown in TJY broth (method 2) was 25. Of the 171 suspensions resulting from these washings, 12 showed a fermentation change—4 changes in cultures grown in semisynthetic medium (one for each single-cell strain) and 8 in cultures grown in TJY broth (one or two changes for each strain). In single-colony cultures the number of washings of cultures grown in TJY broth (method 2) was 19. Of the resulting suspensions, 7 showed a fermentation change. A change occurred four times in one of the single-colony strains, twice in a second, once in a third, and no change took place in the fourth strain.

The washing process was carried out on 42 separate days over a period of 7 months. During the first 5 months single-cell cultures only were washed. Four washings were generally carried out simultaneously, and these usually involved the preparation of suspensions from all 4 of the single-cell strains. In the first 5 months 144 washings were done on 33 days. (Four of these were washings of cultures grown in TJY broth as part of the procedure described as method 2. All the rest were of cultures grown in semisynthetic medium as described in method 1.) During this time no fermentation changes occurred. During the subsequent two months both single-cell and single-colony cultures were washed. In this period 46 washings were done on 9 days. (Six of these washings were of single-cell cultures grown in semisynthetic medium as described in method 1. The rest were of cultures grown in TJY broth—method 2.) On 8 of these days fermentation changes were found. The incidence of change is presented in table 1.

TABLE 1.—*Fermentation changes in oral lactobacilli.*

Date	Number of cultures washed			Number of cultures changed		
	Single-cell	Single-colony	Total	Single-cell	Single-colony	Total
1-20-50	6	2	8	3	1	4
1-27-50	2	0	2	0	0	0
1-30-50	2	0	2	2	0	2
2- 8-50	2	2	4	1	2	3
2-24-50	2	2	4	2	2	4
3- 3-50	2	2	4	1	1	2
3- 9-50	4	4	8	0	1	1
3-17-50	3	3	6	1	0	1
3-28-50	4	4	8	2	8	2

The six washings of single-cell cultures grown in semisynthetic medium were done on 1-20, 1-27, and 1-30, two on each day. Of these 6, the 4 changes in fermentation occurred on 1-20 and 1-30, two on each day.
On 1-20 cultures of the same single-cell strain grown in semisynthetic medium and TJY broth were washed simultaneously, and both resulting suspensions showed changed fermentation.

During the period of two months in

which the fermentation changes in washed cultures occurred, half of the 74 transfers with no washing, described in method 3, were made—19 of the 56 transfers of single-cell cultures and all 18 of the transfers of single-colony cultures.

Of the total 19 changes, 12 were complete when first noted—i.e., fermentation of both sorbitol and mannitol (accompanied by the fermentation of milk), and 7 were partial changes—i.e., fermentation of either sorbitol or mannitol but not of the other (and with or without fermentation of milk). All of the partial changes became complete after subculture.

Of the four changes in single-cell strains which took place in cultures grown in semisynthetic medium, all occurred in washed suspensions from thiamin-containing medium; none in suspensions from the medium containing no thiamin. Good growth of the unchanged organisms often occurred after a transfer from thiamin-containing medium into thiamin-free medium. This was apparently because of a carry-over of thiamin by the washed cells, since growth never occurred after transferring a non-fermenting culture from thiamin-free medium to a second tube of this medium. Following the change in fermentation, the changed cultures grew well in thiamin-free medium, and serial transfers (with intervening washing) in this medium were successfully made.

DISCUSSION

The nonrandom distribution of the changes and their association with washing have been described previously.[2] There appears to be some factor associated with the washing process which increases the rate of change. The coincidence of changes on the same days in different strains and in cultures grown in different mediums following repeated washings with no change suggests an unknown variable in the environment or technique as an element essential to the change.

The failure of thiamin-free medium to promote the change in the organisms apparently differentiates the mechanism involved here from that involved in the loss of thiamin requirement which has been observed in other organisms when grown in thiamin-free mediums.[9]

The fact that changes occur in cultures derived from single cells shows that the change is not due to selection from an originally heterogeneous population. This change, occurring with an artificially increased frequency associated with the washing process, is reasonably certainly identical with the changes frequently observed by Harrison[1] to occur spontaneously in single-colony cultures.

SUMMARY

The fermentation changes in oral lactobacilli originally described by Harrison have been shown to occur in cultures derived from single cells.

The incidence of change has been observed to be directly associated with washing of the organisms in 0.85% sodium chloride solution.

9. Wood, H. G., Anderson, A. A. and Werkman, C. H. 1938, J. Bact. **36**: 201–214.

HETERO- TO HOMOFERMENTATIVE CHANGE IN LACTOBACILLI

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This paper describes the evidence that the change in fermentation characteristics of the single-cell cultures of oral lactobacilli described in the previous paper (Hodges et al, 1951) is accompanied by a change from the heterofermentative to the homofermentative type of metabolism (the former type of metabolism yielding one mole of carbon dioxide, one mole of lactic acid and various amounts of other intermediates per mole of glucose; the latter type yielding practically quantitatively two moles of lactic acid per mole of glucose). The paper also describes a simple method of measuring the carbon dioxide output of small amounts of lactobacilli.

EXPERIMENTAL

Cultures.—The cultures used were derived from the single-cell cultures described in the previous paper (Hodges et al, 1951), the latter prepared from the cultures EH68H and RH37E of oral lactobacilli originally isolated by Harrison. As described in the previous paper in detail these single-cell cultures were maintained in Jay's broth and tomato-juice yeast-extract agar for from 2 to 4 years in a state in which the cultures did not ferment sorbitol and mannitol and were then subjected to serial transfers, with washing, in semisynthetic mediums, giving rise to cultures which did ferment sorbitol and mannitol.

The lactobacilli were examined (a) as removed from unchanged subcultures prepared from stock tomato-juice yeast-extract broth; (b) as removed from changed cultures, the change in fermentation of which had been maintained during at least three successive transfers through semisynthetic mediums and tomato-juice yeast-extract broth.

Mediums.—The mediums used were (a) tomato-juice yeast-extract (Orland, 1946), and (b) a modification of the synthetic medium of Henderson and Snell (1948), the modification being: (1) the replacement of the mixture of individual amino acids by casein hydrolysate and *L*-tryptophan to give 1 g of casein hydrolysate (General Biochemicals) nitrogen per liter and 320 mg of *L*-tryptophan per liter; (2) replacement of sodium citrate and sodium acetate by 33 ml of 4M KH_2PO_4-K_2HPO_4 buffer, pH 7.2, per liter of medium; (3) the elevation of the glucose concentration to 2%; (4) a 30-fold elevation in the vitamin concentration.

Analytical methods.—Glucose was determined by the Shaffer-Hartman-Somogyi method; carbon dioxide by the Van Slyke-Neil method, both methods as described by Koch and Hanke (1948). Lactic acid was determined by the Barker and Summerson (1941) method and acid production was measured by titration.

Procedures.—Cultures attaining full growth, usually within 48 hours, in tomato-juice yeast-extract broth, were washed three times in the centrifuge with 0.85% sodium chloride and part or all of the suspensions inoculated into 5 ml of semisynthetic medium in Thunberg tubes autoclaved 4 minutes at 20 lb with loose cotton plugs in female joint and side arms. Into each of the upper parts of the Thunberg tubes was pipetted 0.5 ml of 5 *N* sodium hydroxide, the male parts of the joints were greased with heavy vacuum grease and the upper parts, after autoclaving as above, were joined to the lower parts after removal of the cotton plugs in the joints. The tubes were evacuated with a water pump, closed and incubated 3 to 6 days to approximately maximal growth. The tubes were then opened, the alkali containing the evolved carbon dioxide was rinsed into 25 ml volumetric flasks; the contents of the lower parts of the Thunberg tubes into 10 ml volumetric flasks and the flasks made up to volume. Aliquots from the flasks were used for analysis.

RESULTS

As can be seen from table 1, both cultures before the change produced one mole of lactic acid and one of carbon dioxide per mole of glucose, and after the change, two moles of lactic acid and negligible amounts of carbon dioxide per mole of glucose.

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TABLE 1.—*Changes in metabolic pattern of lactobacilli.*

Culture	State	Metabolites, millimoles/tube			
		Glucose used	Lactic acid produced	CO_2 produced	Acid by titration
EH68	Before change	0.55	0.62	0.50	0.65
"	"	0.56	0.52	0.58	0.50
"	After change	0.54	1.01	0.03	1.00
"	"	0.54	1.09	0.03	1.01
RH37	Before change	0.56	0.52	0.41	0.65
"	"	0.56	0.54	0.33	0.40
"	After change	0.56	1.19	0.03	1.01
"	"	0.52	1.01	0.03	0.90

DISCUSSION

The change in metabolic activity from the hetero- to homofermentative type is accompanied as has been shown in the previous paper by a loss in thiamin requirement. This fact is consistent with the generalization that the heterofermentative lactobacilli require thiamin and the homofermentative lactobacilli do not require thiamin.

A few analyses of thiamin production and consumption indicate that the homofermentative organisms are able to produce thiamin (an external source of which they do not require) but that the rate of thiamin production by the homofermentative organisms is less than the rate of consumption of thiamin by the heterofermentative ones. The homofermenters show practically no destruction of thiamin. Subculture of the heterofermenters in thiamin deficient mediums does not increase the incidence of change.

The method of collecting and measuring the carbon dioxide evolved is quantitative and sensitive. If the Thunberg tubes are adapted to use with a photoelectric colorimeter, the method allows simultaneous measurement of turbidity of the culture.

SUMMARY

The change in lactobacilli originally observed by Harrison as the acquisition of the ability to ferment sorbitol and mannitol has been shown in single-cell cultures to involve a change from the heterofermentative to the homofermentative type of metabolism.

A method of collecting and measuring the carbon dioxide evolved by small amounts of bacteria grown in Thunberg tubes is described.

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STUDIES ON THE EXPERIMENTAL EPIDEMIOLOGY OF RESPIRATORY INFECTIONS

V. EVALUATION OF FACTORS RELATED TO SLIT SAMPLING OF AIR-BORNE BACTERIA

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A method of evaluating the bacterial content of air is to direct a measured sample of the air moving at high velocity through a slit or jet against a nutrient agar surface. A count of the colonies appearing after suitable incubation gives a quantitative picture of the bacterial content of the air sample. Using a "slit sampler," Bourdillon et al[1,2] recovered more than 90% of bacteria from aerosols. In our experience, air velocities higher than those used by Bourdillon et al were found to be detrimental to certain organisms. It is the purpose of this communication to present the results obtained in evaluating the effect of air velocity, slit width, and slit to agar distance upon sampling of bacterial aerosols. Further application of this method allows the determination of the median diameter of the aerosol particles containing viable bacteria.

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The opinions and assertions contained in this report are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. Article 1252, U. S. Navy Regulations 1948.

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1. Bourdillon, R. B., Lidwell, O. M. and Thomas, J. C. 1941, J. Hyg. **41**: 197–224.
2. Bourdillon, R. B. et al. 1948, Studies in air hygiene. Medical Research Council Special Report Series #**262**: 12–50. His Majesty's Stationery Office, London.

MATERIALS AND METHODS

An instrument was constructed to allow variation of slit width (by provision of air inlet tubes with slits of varying sizes), clearance between the slit and the collecting surface, and air velocity. Figure 1 illustrates how these factors were introduced into the design of the instrument.

Aerosols of *Streptococcus zooepidemicus* and *Pasteurella pestis* were produced in the apparatus described by Leif and Krueger.[3] This apparatus consists, essentially, of a Wells type nebulizer, a mixing chamber allowing the introduction of additional air for humidity control, and a chamber designed for the exposure of small animals to the prepared aerosol. The slit sampler was attached to the sampling port. Aerosols were generated by spraying a suitable dilution of the culture prepared in the same medium as was used for growth, namely tryptose-phosphate-infusion broth for *Str. zooepidemicus* and heart infusion broth for *P. pestis*. All runs were made at room temperature and at a humidity of approximately 40%. Based on the volume of the exposure unit, the traverse time of the aerosol was 15 seconds from source to collecting port. The desired volume of the aerosol was directed against the agar surface (1% horse blood for *P. pestis* and 3% cow blood agar for *Str. zooepidemicus*) which was rotated at one revolution per minute. The number of impinged organisms was determined by colony count following incubation at 37 C for 24 to 48 hours. The sampling efficiency of the collecting unit was tested for various adjustments of slit width, slit to agar clearance, and air velocity.

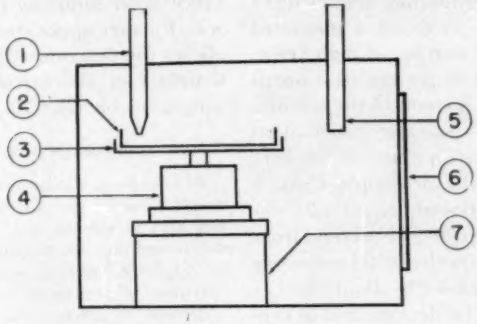
3. Leif, W. R. and Krueger, A. P. 1950, J. Infect. Dis. **87**: 103–116.

RESULTS AND DISCUSSION

In the report by one of us (L. J. G.) on the determination of the particle size distribution of air-borne bacteria[4] and in an earlier report by May[5] it was demonstrated that when a jet of air was directed normally against a suitable surface, particles of a diameter "D_{50}" or larger could be efficiently removed by impaction. On the basis of the above work[4,5] a general equation was found relating D_{50} to the conditions of sampling. This is given as:

$$D_{50} = \frac{kx}{v\rho} \text{ microns}$$

where k = a constant, which for the apparatus used in this work was 76, x = slit width in mm, v = jet air velocity, in meters per second, and ρ = density of particle. This suggests that one might describe the behavior of a slit sampling device in terms of a particle size D_{50}. It was convenient to call the size D_{50} associated with a given set of sampling conditions the "slit index." Since it was found to be detrimental in general in sampling to operate beyond 1 to 2 mm clearance, it was felt that no alteration need be made in formula 1 for increasing the slit clearance beyond this range.



1. AIR SAMPLING TUBE (JET 0.015 CM. x 0.938 CM. OR 0.028 CM. x 0.938 CM.)
2. PETRI DISH CONTAINING AGAR
3. PETRI DISH PLATFORM
4. 1 RPM SYNCHRONOUS MOTOR
5. AIR EFFLUENT TO VACUUM LINE
6. SEALED CONTAINER WITH QUICK ACTION DOOR
7. SCREW MECHANISM FOR ELEVATING PLATFORM

FIG. 1.—Schematic diagram of slit air sampler.

The results of experiments with *Str. zooepidemicus* and *P. pestis* are summarized in figures 2 and 3. Typical experimental data are given in tables 1 to 3. It is seen that optimum sampling was obtained for all values of the slit index between (a) 0.2 and 0.7 μ in the case of *Str. zooepidemicus* and (b) 0.4 to 0.8 μ for *P. pestis*. In the case of *P. pestis*, a drop in recovery to 40% of the expected value resulted for a slit index of 0.18 μ, using a slit to agar distance of 1 to 2 mm; with *Str. zooepidemicus* under similar conditions no comparable effect was observed. The

4. Goldberg, L. J. 1950, J. Infect. Dis. **87**: 133–141.
5. May, K. R. 1945, J. Sci. Instr. (London) **22**: 187–195.

TABLE 1.—*Comparative cloud recovery of an aerosol of* Str. zooepidemicus *under various sampling conditions at slit-agar distance of 1 to 2 mm.*

	Sampling rate, L/min: 7.3 Slit width, cm: 0.028 Sampling time, min: 3.0		15.0 0.028 1.5		19.0 0.015 1.0	
	Sample no.	Viable organisms recovered	Sample no.	Viable organisms recovered	Sample no.	Viable organisms recovered
Series I	1	192	3	168	5	127
	2	158	4	144	6	X
	7	199	9	X	11	153
	8	214	10	215	12	151
	13	103	15	181	17	112
	14	194	16	206	18	136
Average number of bacteria/L of air		8.1		7.8		7.2
Series II	1	163	3	128	5	100
	2	147	4	107	6	72
	7	127	9	140	11	94
	8	113	10	129	12	115
	13	206	15	151	17	166
	14	206	16	163	18	159
Average number of bacteria/L of air		7.3		6.0		6.2
Series III	1	200	3	166	5	96
	2	159	4	145	6	111
	7	84	9	95	11	82
	8	78	10	108	12	89
	13	102	15	91	17	73
	14	105	16	97	18	76
Average number of bacteria/L of air		5.5		5.0		4.6

X = contaminated.

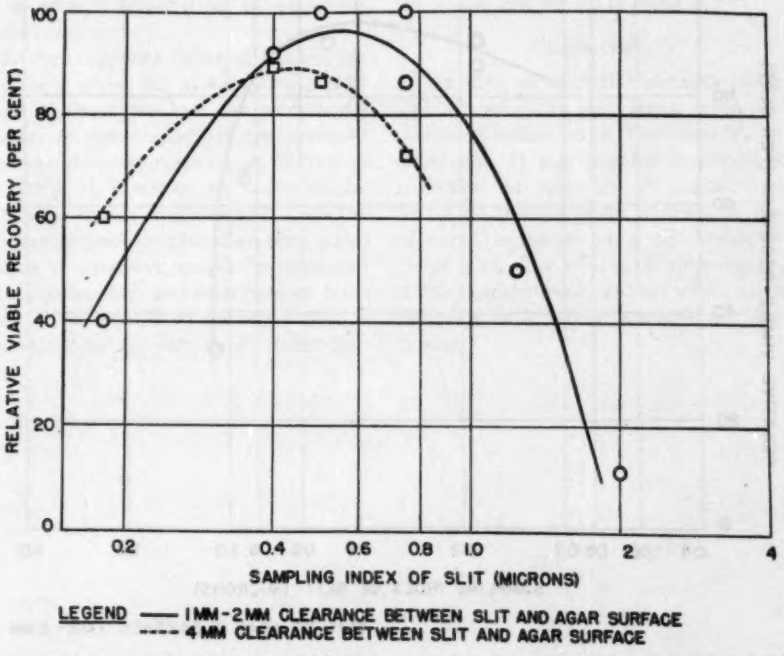


FIG. 2.—Relative viable slit-sampler efficiency evaluated for an aerosol of *P. pestis* having a mean diameter of 1.3 μ.

TABLE 2.—*Illustrative protocol of comparative viable recovery of* P. pestis *aerosol sampled for 2 minutes at 19 L per minute at varied slit-agar distances through the 0.015 cm slit.*

1 mm		2 mm		4 mm	
Sample no.	Viable organisms recovered	Sample no.	Viable organisms recovered	Sample no.	Viable organisms recovered
1	193	3	193	5	294
2	170	4	197	6	224
7	188	9	184	11	241
8	174	10	160	12	244
13	163	15	185	17	X
14	155	16	143	18	310
19	175	21	217	23	256
20	207	22	192	24	255
25	129	27	176	29	214
26	158	28	198	30	238
Average recovery	171		185		252
Relative % recovery	68		73		100

X = contaminated.

TABLE 3.—*Illustrative protocol of comparative viable recovery of* P. pestis *aerosol sampled at 19 L per minute through 0.028 and 0.015 cm slits, at slit-agar distance of 1 to 2 mm.*

Slit width of .028 cm		Slit width of 0.015 cm	
Sample no.	Viable organisms recovered	Sample no.	Viable organisms recovered
1	420	4	148
2	356	5	145
3	364	6	106
7	237	10	70
8	217	11	72
9	200	12	X
13	187	16	49
14	176	17	61
15	153	18	48
Relative % recovery	100		40

X = contaminated.

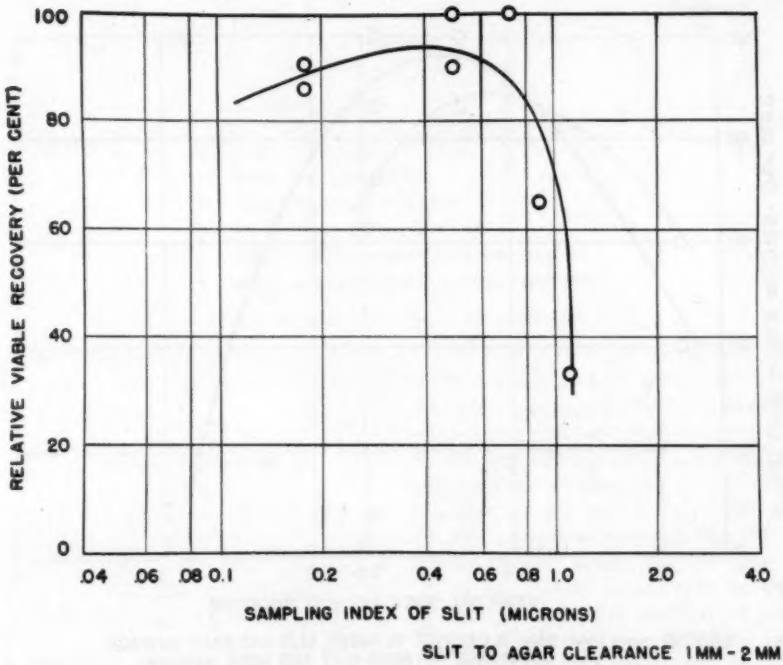


FIG. 3.—Relative viable slit-sampler efficiency evaluated for an aerosol of *Str. zooepidemicus* of 1.1 μ mean particle diameter.

decreased recovery of *P. pestis* may be attributed to a fracture or shattering of the bacterial cells.

Under the above conditions of sampling, using a slit index of 0.18, increased recovery of *P. pestis* occurred when the slit to agar distance was 4 mm, since the widening of the air jet before its impaction against the agar may have decreased the possibility of shattering. Using slit indices of 0.4 to 0.8, sampling was less efficient when the slit to agar distance was increased to 4 mm. The data presented in figures 2 and 3 further indicate that, using a given slit, a tenfold range in sampling rate may be used for bacterial aerosols. Correspondingly, this suggests that organisms varying in diameter by a factor of 3 may be sampled with equal efficiency, but greater variation in size should lead one to a critical evaluation of sampling technique.

If the computed value of the slit index for a given set of sampling conditions is about one-half the median diameter of the droplets in the aerosol,[5] one would expect recovery in the region of 100%. If, however, the value of the slit index is very much smaller than the median aerosol droplet diameter, a decrease in recovery might be expected due presumably to shattering or fracture of the contained bacterial cell, as evidenced in the case of *P. pestis* (fig. 2) at a slit index of 0.18.

It is possible to estimate the median particle size by plotting the relative recovery from the aerosol as a function of the slit index (figs. 2 and 3). The median particle size may be considered as the largest value of the slit index associated with a recovery of 50% of the experimentally determined maximum.

It has been found that slits of a width smaller than 0.025 cm are difficult to maintain clean, even under laboratory conditions, and consequently should be avoided whenever possible. Slits may be used with sampling rates of greater than 1 cu ft (28.3 L) of air per minute and still maintain high sampling efficiency. If the capabilities of this type of sampling instrument are utilized to their fullest extent, it is possible to sample aerosols of concentrations as low as one viable cell per cu ft of air.

SUMMARY

Sampling of bacterial aerosols using high air velocities may result in an appreciable reduction in the recovery of organisms. It was possible to obtain a reduction in recovery of viable airborne *Pasteurella pestis* to 40% of the expected optimum at a slit index of about 0.18. The effects of air velocity, slit to agar distance, and slit width upon sampling of bacterial aerosols are discussed.

STUDIES IN TRICHINOSIS

I. IMMUNITY TO REINFECTION IN MICE FOLLOWING A SINGLE LIGHT INFECTION

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Reports regarding resistance to reinfection with *Trichinella spiralis* have been somewhat contradictory. McCoy (1931, 1940) used hyperimmune rats (three or more larval immunizing doses), and found that most of the larvae were expelled within 3 to 18 hours after feeding. Roth (1939), working with guinea pigs after a single reinfection, found no large initial loss of worms until the 8th to 10th days, and that the worms completely disappeared between the 13th and 20th days. Culbertson (1942) reported that 5 days after a second infection in mice, only 9% of larvae fed were recovered as adults. Larsh and Fletcher (1948) found that mice killed 7 days after a single reinfection, showed no evidence of immunity to adult worms.

In view of the conflicting results of various workers we felt it worthwhile to reinvestigate the question of immunity using several of the quantitative techniques previously reported (Rappaport, 1943 a and b).

METHODS AND MATERIALS

The experimental animals were male mice weighing approximately 20 g. All larvae for infection were obtained from rats infected 2 to 5 months previously. Infecting doses were counted microscopically and administered by means of a fine pipette inserted into the esophagus. In each experiment, one reinfected and one infected control mouse were killed at intervals during the intestinal phase, and adult worms in the entire intestine were counted directly. Larval counts in the musculature were determined in mice killed at least 45 days after infection by averaging 10 dilution counts of the entire digested carcass. Only those mice were used which survived the infection and from cages where there was no evidence of cannibalism.

Adult worms were measured in those experiments where the time interval between infections was sufficiently long to insure the loss of adult worms of the initial infection. After counting the adults in the intestine, the worms were immobilized with a minimal amount of Lugol's iodine solution. The images of the worms were then projected on a screen with a 100× magnification by means of a compound microscope and a reflecting prism. Lengths were obtained to the nearest tenth of a mm, using a map measure. These measurements were made of the first 10 intact worms of each sex encountered.

In three instances on initial infection, there was a consistent, unexplained, early loss of larvae or adults from the intestines of mice, similar to that previously reported (Rappaport, 1943 a). It is felt that this decrease in infectivity may have resulted from some alteration in the larvae prior to infection of the mice. Since it would not have been possible to relate such lower grade initial infections with those of other experiments where the same larval dosage resulted in a larger adult worm yield, each of these series was immediately discarded.

EXPERIMENTAL

In all instances, mice were given an initial infection with 100 ± 5 larvae. Single reinfecting doses consisted of either 800 ± 25 or 300 ± 10 larvae. Challenging doses were given at time intervals of 10, 35, and 36 days and 3.5

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Preliminary trials not reported herein were conducted in the Department of Public Health and Preventive Medicine, Cornell University Medical College, New York City.

months. Control animals were similarly infected.

Numbers of adults recovered from the intestines.—(Figs. 1 to 3B; figure numbers correspond to experiment numbers.) Since the normal loss of worms occurs between the 16th and 18th day after infection (Rappaport, 1943 b), the course of the intestinal phase was not followed much longer than this period. A change occurring during this three-day interval can be attributed to the normal loss of worm burden. In experiment 1 (800 larvae, reinfected after 10 days) there was practically no difference between test and control animals in the number of adult worms recovered. In experiment 2A (800 larvae, reinfected after 3.5 months) there appeared to be a slight reduction in the number of adult worms after the 4th day, but the differences were not striking. In experiment 2B (a duplicate of 2A in which reinfections were performed one day later with larvae from another rat) reinfected animals showed no indication of a reduction in adult worm counts before the 17th day. In experiment 3A (800 larvae, reinfected after 36 days) there was no appreciable difference between reinfected and control mice until the 14th day, when fewer adults were recovered from reinfected mice, and no worms were present on the 18th and 20th days. In experiment 3B (300 larvae reinfected after 35 days) there were striking differences in the number of worms recovered from the 8th day through the course of the infection.

Initially, there are about twice as many female as male worms. In previous investigations (Rappaport, 1943 b) we found that by approximately the 17th day after infection, when the adult phase begins to terminate, female worms are lost more rapidly than males, resulting in a reversal in the sex ratio. In the present experiments the changes in the sex ratios of reinfected animals were similar to those of controls.

Length measurements of adult worms.—Adult worms were not measured in experiment 1, since reinfected mice still harbored adults of the infection 10 days previous. Lengths of adult male and female worms from the other experiments are tabulated in table 1. Worms did not attain their maximum lengths until after the 4th day.

In table 1, lengths of both male and female worms from 55 pairs of animals are compared. In 49 of these, the control animals showed a larger average worm length than the reinfected animals. This result is clearly significant. Of the 6 instances where average worm lengths of reinfected animals were larger than in controls, none was significant, 4 occurred among male worms, and 4 occurred in experiment 2B.

It was observed that the uteri of female worms from reinfected animals appeared less filled and contained fewer eggs and embryos than female worms from control animals.

Larval counts in the musculature.—(table 2). The larval counts in the musculature of reinfected animals may be considered to represent a sum of two infections. In order to compare larval yields resulting from reinfection with the yields from corresponding control animals, it appears justifiable, unless other possible factors exist, to subtract the number of larvae contributed by the initial infection from the total larval yields obtained after reinfection. Thus in experiment 1, the average number of larvae contributed by the second infection alone would be 123,000 (i.e., 203,000 − 80,000); since the control animals yielded 232,000 the larval yield resulting from reinfection was therefore 53% of that of the controls. Similarly for experiment 2A the yield was 38% of

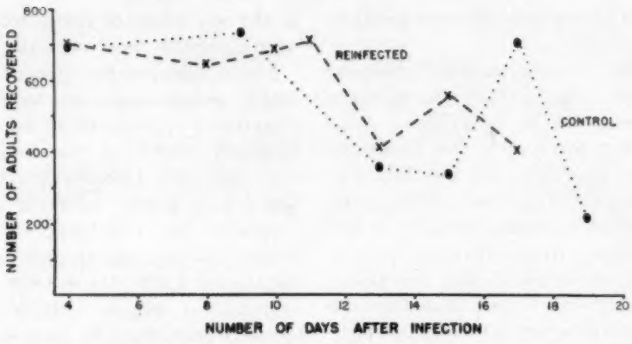

FIG. 1 (exp. 1).—Longevity of *T. spiralis* adults in mice after an initial infection of 100 larvae and reinfection after 10 days with 800 larvae, as compared with a single infection of 800 larvae in controls. Each point in figures 1, 2A, 2B, 3A and 3B represents the total number of adult worms recovered from a single mouse.

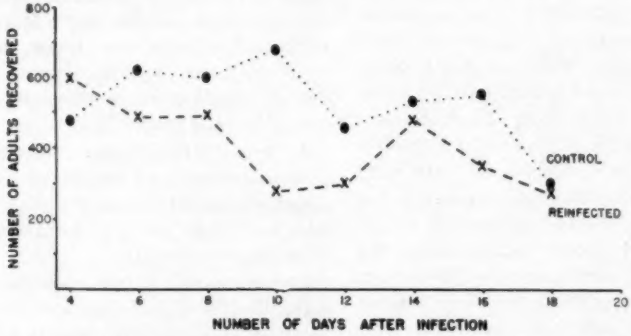

FIG. 2A (exp. 2A).—Longevity of *T. spiralis* adults in mice after an initial infection of 100 larvae and reinfection after 3.5 months with 800 larvae, as compared with a single infection of 800 larvae in controls.

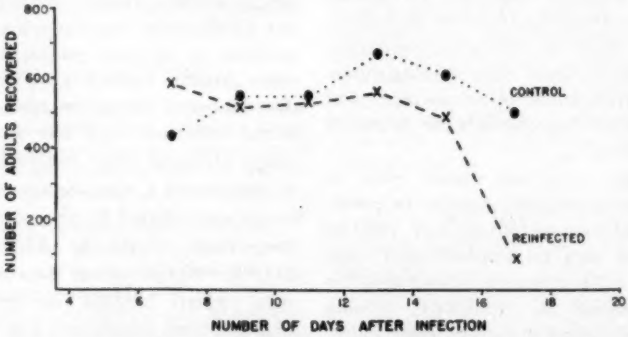

FIG. 2B (exp. 2B).—Longevity of *T. spiralis* adults in mice after an initial infection of 100 larvae and reinfection after 3.5 months with 800 larvae, as compared with a single infection of 800 larvae in controls. This is a duplicate of experiment 2A.

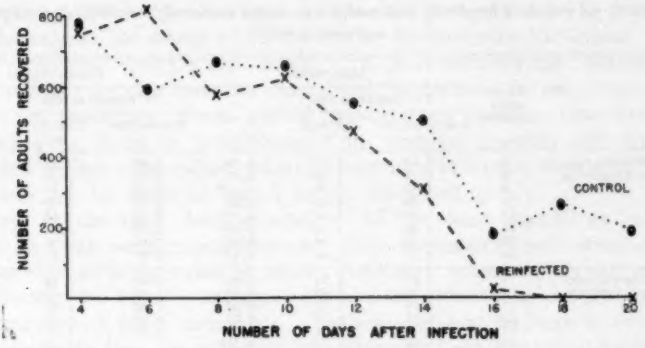

FIG. 3A (exp. 3A).—Longevity of *T. spiralis* adults in mice after an initial infection of 100 larvae and reinfection after 36 days with 800 larvae, as compared with a single infection of 800 larvae in controls.

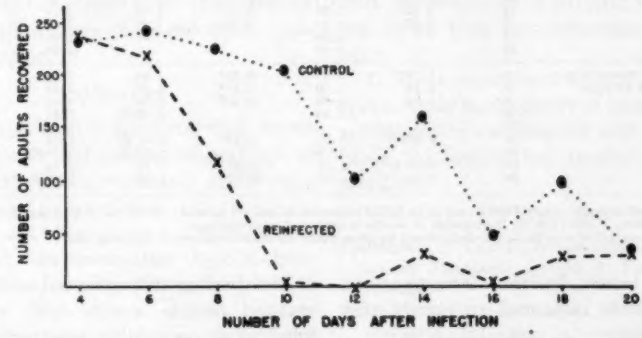

FIG. 3B (exp. 3B).—Longevity of *T. spiralis* adults in mice after an initial infection of 100 larvae and reinfection after 35 days with 300 larvae, as compared with a single infection of 300 larvae in controls.

the controls; 2B, 56%; 3A, 16%; and 3B, 7%.

DISCUSSION

Our results on adult worm yields after a single reinfection cannot be compared with those of McCoy (1931, 1940), since he used hyperimmunized rats. We did not, however, find any indication of a rapid mechanical expulsion of fed larvae or adult worms.

It is significant that immunity was best exhibited in mice which were reinfected after 35 days with a smaller dose of larvae (300 instead of 800). This indicates that immunity may be better manifested against smaller challenging doses of larvae and that when large, although sublethal doses are given, the infection may break through existing immunity. This may explain divergent results between various reports in which mice were used as experimental animals. Culbertson's (1942) low adult worm yields 5 days after reinfection may have resulted from the smaller reinfecting dose (100 larvae initially and 175 larvae for reinfection 22 days later). The findings of Larsh and Fletcher (1948) that there was no reduction in adult worm counts 7 days after reinfection might be explained on the same basis (300 larvae initially and 900 larvae for reinfection

TABLE 1.—*Analysis of lengths of male and female worms recovered from individual reinfected and control mice.*

	Days after infection	Male worms			Female worms		
		Av. lengths in mm			Av. lengths in mm		
		Reinfection x_1	Control x_2	x_2-x_1	Reinfection x_1	Control x_2	x_2-x_1
Exp. 2A: 800 larvae reinfected after 3.5 months	4	1.68	1.63	−0.05	2.80	2.85	0.05
	6	1.74	1.78	0.04	2.72	3.33	0.61*
	8	1.64	1.76	0.12*	2.55	3.57	1.02*
	10	1.59	1.68	0.09	2.25	3.34	1.09*
	12	1.67	1.84	0.17*	2.42	3.38	0.96*
	14	1.45	1.73	0.28*	2.33	3.11	0.78*
	16	1.58	1.58	0.00	2.15	2.53	0.38*
	18	1.56	1.69	0.13	1.86	2.31	0.45*
Exp. 2B: 800 larvae reinfected after 3.5 months	7	1.57	1.54	−0.03	2.45	3.14	0.69*
	9	1.70	1.65	−0.05	2.68	2.62	−0.06
	11	1.76	1.73	−0.03	2.60	2.80	0.20
	13	1.75	1.77	0.02	2.58	3.10	0.52*
	15	1.70	1.63	0.07	2.27	2.33	0.06
	17	1.56	1.74	0.18*		3.15	
Exp. 3A: 800 larvae reinfected after 36 days	4	1.50	1.55	0.05	2.45	2.68	0.23
	6	1.51	1.67	0.16*	2.82	3.34	0.52*
	8	1.62	1.70	0.08	2.11	2.73	0.62*
	10	1.47	1.71	0.24*	2.52	2.79	0.27
	12	1.59	1.71	0.12	2.42	2.97	0.55*
	14	1.57	1.58	0.01	2.08	2.77	0.69*
	16	1.40(3)	1.52	0.12	1.87	2.20	0.33*
	18		1.66			2.40	
	20		1.63			2.84	
Exp. 3B: 300 larvae reinfected after 35 days	4	1.29	1.49	0.20*	1.94	2.56	0.62*
	6	1.44	1.60	0.16*	1.96	3.12	1.16*
	8	1.62	1.78	0.16*	2.41	3.46	1.05*
	10		1.64		2.45(2)	3.53	1.08*
	12					2.50(1)	
	14	1.55(3)	1.72	0.17*	2.47(6)	3.03	0.56*
	16		1.79		1.82(5)	2.51(7)	0.69*
	18	1.70(4)	1.77	0.07	2.79	2.74	−0.0
	20	1.51	1.53	0.02	1.99	2.90(5)	0.91*

All reinfected animals received 100 larvae as an initial infection. Whenever possible, 10 worms of each sex were measured from each animal; if less than 10, the number of worms is indicated in parenthesis.
* Indicates statistical significance as determined by three times the standard error of the difference.

35 days later). Further investigations however, are indicated to clarify this possibility.

In a previous report (Rappaport, 1943 b), it was suggested that the reversal in sex ratio in favor of the male worms might be used to study the immunity in trichinosis. This could not be demonstrated in the experiments reported herein.

The finding in reinfected animals of stunted female worms with sparsely filled uteri containing relatively fewer embryos indicated that immunity plays a role in their development. Male worms were also stunted but not to as significant a degree.

The results of these experiments demonstrate that immunity in trichinosis may be manifested in several ways.

TABLE 2.—*Results of larval counts obtained after pepsin-hydrochloric acid digestion.*

Exp. number		Initial infection (dose: 100 larvae)			Reinfection (doses: 100 larvae and 800 larvae)				Control (dose: 800 larvae)		
		No. of mice used	Av. no. of larvae recovered	Standard deviation	Interval between infections	No. of mice used	Av. no. of larvae recovered	Standard deviation	No. of mice used	Av. no. of larvae recovered	Standard deviation
1		14	80,000	25,000	10 days	10	203,000	53,000	7	232,000	47,000
2	A	20	62,000	20,000	3.5 months	15	162,000	40,000	9	260,000	63,000
	B					14	164,000	40,000	11	183,000	26,000
3	A	18	51,000	17,000	36 days	23	92,000	33,000	7	252,000	44,000
					(Doses: 100 larvae and 300 larvae)				(Dose: 300 larvae)		
	B				35 days	23	60,000	23,000	24	135,000	46,000

Quantitative studies of adult worm yields throughout the course of the intestinal infection, lengths of adult worms, and larval yields from the musculature are important criteria. All of these, however, must be investigated and related to each other before definite conclusions can be made in regard to immunity. It has been demonstrated that after a single reinfection, although there may not be a reduction in adult worm burden, the females are stunted in size and contain fewer embryos.

It is probable that the recovery of fewer larvae from the musculature of reinfected mice is more the result of diminished fecundity of the female worms than their early expulsion from the intestine.

SUMMARY

1. Experiments are presented which demonstrate various manifestations of immunity in experimental trichinosis. Mice were infected with 100 larvae initially, and then given a single reinfection with 800 larvae after 10 or 36 days, 3.5 months, or with 300 larvae after 35 days.

2. Adult worm counts during the course of the intestinal phase did not show significant differences between reinfected and control animals when the number of larvae fed was 800, a dose which is just sublethal. When mice were reinfected with 300 larvae 35 days after the initial infection, there was a loss of adult worms beginning early in the second week of reinfection.

3. Adult male and female worms were significantly shorter in length in reinfected animals. The uteri of these female worms appeared sparsely filled with eggs and embryos.

4. The larval phase in the musculature for the 800 larvae infections indicated the lowest immunity after 10 days and the highest immunity after 36 days. The most striking manifestations of immunity were obtained in reinfections after 35 days with 300 larvae.

5. It is believed that the low larval yield in the musculature, in spite of high adult worm yields, results mainly from the stunted growth and diminished fecundity of female worms in intestines of reinfected mice.

6. The least significant manifestations of immunity were obtained with a duplicate series reinfected after 3.5 months with 800 larvae. The reason for these atypical findings is not known. These and other atypical findings such as consistently poor adult yields in previously uninfected animals indicate that the degree of infectivity of larvae may vary from one experiment to the next.

7. The utilization of several quantitative criteria in the study of immunity in trichinosis is emphasized and may explain the conflicting results of other workers.

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RAPID DIFFERENTIATION BETWEEN *PASTEURELLA PESTIS* AND *PASTEURELLA PSEUDOTUBERCULOSIS* BY ACTION OF BACTERIOPHAGE

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In the identification of *Pasteurella pestis*, differentiation from *Pasteurella pseudotuberculosis* may often be difficult particularly in cultures obtained from rodents. A bacteriophage which lysed *P. pestis* but not the strain of *P. pseudotuberculosis* tested was isolated by Sugino.[1] In previous reports from this laboratory a bacteriophage for *P. pestis* has been described which also lysed many strains of *P. pseudotuberculosis*.[2,3] The activity of this phage against *P. pseudotuberculosis* was less marked at 20 C than at 37 C.[4] The present report describes a method of differentiating the two species of pasteurellae by the action of this phage at room temperature.

MATERIALS AND METHODS

Bacteriophages.—The phage of Advier[5] was adapted to a strain of avirulent *P. pestis* (A 1122) and is designated as "P phage." This P phage was adapted to a strain of *P. pseudotuberculosis* (Spokane) against which it originally had little action and is designated as "PTB phage."

Bacterial cultures.—The strains of *P. pseudotuberculosis* tested have been described.[3,4] Five additional strains, 2 from human cases, were obtained from the Hygiene Institute of the University of Zurich, making a total of 45 strains. Seventeen avirulent and 35 virulent strains of *P. pestis* from the collection of the Hooper Foundation were tested.[6]

Technique.—Plates containing 20 ml of proteose #3 agar (Difco) were allowed to dry overnight in the incubator and then for several days at room temperature. The bacterial cultures were grown in broth for 18 to 24 hours at room temperature (18 to 20 C). The cultures were spread with a loop having an inside diameter of 3 mm over circular areas 2 cm in diameter on the surface of the dry plates taking care not to scratch the agar. As soon as the inoculum had dried, a loopful of phage was touched to the center of the area being careful not to disturb the film of organisms. When the phage had dried, the plates were inverted and held either at 37 C or at 20 C for 48 hours. As many as 10 tests could be made on a single plate.

Each phage was first titrated against the culture to which it had been adapted. Serial tenfold dilutions of phage were tested as described above. The titrations of P phage were held at room temperature and those of PTB phage were incubated at 37 C. The highest dilution giving confluent lysis was recorded. For the final tests, 10 times this amount of phage was used and designated as the "critical test dilution."

RESULTS

The results are summarized in table 1. The PTB phage was of no value in separating the two species; most of the *P. pestis* cultures were lysed at either 37 C or 20 C, and most PTB cultures were not lysed at 20 C. The P phage at 37 C lysed many strains of *P. pseudotuberculosis* and failed to lyse a few strains of *P. pestis*, so that the two species could not be differentiated by phage action at this temperature. At 20 C,

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however, the P phage was completely specific for *P. pestis*.

TABLE 1.—*Lysis of* P. pestis *and* P. pseudotuberculosis *by critical test dilutions of phages.*

Cultures tested	No. of strains tested	No. of strains lysed by *P. pestis* (P) phage		No. of strains lysed by *P. pseudotuberculosis* (PTB) phage	
		37 C	20 C	37 C	20 C
P. pseudotuberculosis	45	21	0	45	6
P. pestis avirulent	17	17	17	16	17
P. pestis virulent	35	28	35	30	34

For differentiation of *P. pestis* from *P. pseudotuberculosis* the use of the critical test dilution of P phage at 20 C suffices; all strains of *P. pestis* are lysed under these conditions, and no strain of *P. pseudotuberculosis* is lysed. To identify *P. pseudotuberculosis* cultures it would be advisable to include tests with PTB phage at 37 C.

DISCUSSION

The action at 20 C of high dilutions of the bacteriophage described above permits the differentiation of *P. pestis* from *P. pseudotuberculosis* within 48 hours even with strains which have become avirulent or otherwise atypical. The serologic and metabolic tests available for identification of these organisms, on the other hand, are both more time consuming and less reliable because of variations from strain to strain. For example, the strains of *P. pestis* fermenting glycerin and rhamnose reported by Chen[7] were readily identified by testing with phage. As Girard[8] has stated, although the two organisms are closely related and some variant strains may be difficult to distinguish, they seem to be separate and distinct species reacting specifically with certain phages.

All 52 of the strains of *P. pestis* tested, whether virulent or not, were lysed by the critical test dilution of P phage at room temperature. The 15 strains tested by Sugino[1] also behaved uniformly with phage. However, Couvy[9] found that a phage could be adapted to a given strain of *P. pestis* so that it became specific for that strain. Such phages would be valuable epidemiologic tools. In preliminary tests in this laboratory, such ultraspecificity has not been demonstrated. Studies are in progress to determine (a) whether quantitative differences occur in the action of P and PTB phages against virulent and avirulent strains of *P. pestis;* and (b) whether phage adapted to various strains of *P. pestis* might reveal differences among them such as those shown by *Salmonella typhosa*.[10]

SUMMARY

All strains of *Pasteurella pestis* tested (35 virulent and 17 avirulent) were lysed by a critical test dilution of *P. pestis* phage at 20 C; none of 45 strains of *Pasteurella pseudotuberculosis* was lysed under these conditions. Hence, the two species can be rapidly and reliably differentiated by the use of *P. pestis* phage at 20 C.

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A STUDY OF FRESHLY GENERATED BACTERIAL AEROSOLS OF *MICROCOCCUS CANDIDUS* AND *ESCHERICHIA COLI*

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In a recent paper, Ferry et al (1949) indicated that a photoelectronic counter devised by Gucker (1947) was suitable for determining the particulate concentration in dilute physically homogeneous bacterial aerosols, provided particulate size somewhat exceeded 0.7μ in diameter.

They suggested that such an instrument might, in conjunction with biological methods, supply much needed information concerning the survival of bacteria in air. Physical measurements alone can only give information concerning total particulate concentration but do not distinguish between inert and living matter; similarly, biological methods only serve to measure the concentration of living organisms. Therefore, to estimate changes in viability, comparison of both measurements is essential.

In this paper, we report further studies on freshly generated dilute bacterial aerosols which appeared to be nearly homogeneous with respect to size. The organisms studied, *Micrococcus candidus* and *Escherichia coli*, differ markedly in their behavior in aerosols. The former is rugged and persistent; the latter relatively fragile. It appears to us likely that these differences may be related to differences in structure and possible that similar differences in other organisms may be a factor in determining their ability to produce airborne infection. Proof of this hypothesis demands further study.

Since readers of this journal may be unfamiliar with the first paper cited, and since the results described here were obtained with a somewhat modified photoelectronic apparatus believed to be sufficiently sensitive quantitatively to enumerate all particles of bacterial size, and since, moreover, results depend upon the exact apparatus used, it is briefly described in Appendix A.

METHODS

General

Organisms grown in broth mediums were washed twice and resuspended in distilled water. Subsequently, aerosols generated from the aqueous suspensions were diluted with filtered air to provide a total articulate concentration of 300 to 2000 per L. Viability of suspension was determined by direct bacterial and culture count.

Samples withdrawn from two arms of a Y-tube by means of electrically driven vacuum pumps passed either into the photoelectronic counter or into impinger bubblers partly filled with fluid. The former instrument provided a direct measure of total particulate concentration; total viable concentration was, however, estimated by counting the colonies appearing after incubation of agar plates poured with aliquots of the collecting fluid contained in impingers, and relating these counts to the volume of air passing through the impingers. Further experimental details appear below.

A. *Bacterial suspension*

Suspensions of *M. candidus* and *Esch. coli* were prepared from cultures grown 16 to 18 hours in about 800 ml of clear filtered tryptic digest broth

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to which 0.3% dextrose had been added. With *M. candidus*, growth was at 20 to 25 C (room temperature), with *Esch. coli*, at 37 C. The cultures were centrifuged, resuspended, and washed twice, each time in about 400 ml of distilled water. The aqueous suspension to be atomized was hand-shaken about 30 minutes with glass beads and diluted with sufficient distilled water to produce a final bacterial concentration of the order of 10^8 per ml.

By eliminating all but traces of solute, this procedure gave assurance that particles counted in the aerosols subsequently produced actually represented bacteria, and that size was reasonably uniform. Without this precaution, many particles in the aerosol might contain only dried solute, and would certainly vary in size, thus making subsequent interpretation of results very difficult. Photoelectronic counts of filtered air were always low, as were similar counts of aerosols generated either from distilled water or possible sediment from filtered broth. Although the latter two appeared negligible, an observed background count on filtered air was regularly deducted from gross particulate count.

The total bacterial concentration of aqueous suspensions was estimated by direct count in a chamber; viable concentration was estimated by the method of serial dilution and counting the number of colonies on pour plates after 48 hours of incubation. Both counts are expressed as number per ml. In the case of *Esch. coli*, these determinations were always made both before a run and on the suspension remaining in the atomizer at its conclusion. In the case of *M. candidus*, it soon became apparent that only the latter was significant. For, although the initial suspension contained many clumps, the suspension at the end of a run contained about 70% singlets, some doublets, and a few clumps of three or more bacteria. Percentage viability, or the ratio $\frac{\text{Viable concentration}}{\text{Total concentration}} \times 100$, was derived from these measurements.

B. *Aerosol generation*

Although this and photoelectronic counting, in general, followed the method previously described, certain significant details have been modified. These are described in Appendix A. The particulate concentration ranged from about 300 to 2000 per L.

C. *Collection and enumeration of viable bacteria contained in aerosols*

Except in a few preliminary experiments in which rubber stoppered flasks were used, all-glass capillary jet bubbler samplers of the type described by Rosebury (1947) and generally two in series, served as collectors. The jet, about 1 mm in diameter, was situated about 5 mm from the bottom of the flask. About 25 ml of sterile gelatin solution, buffered at pH 7.0 with potassium phosphate to which a few drops of olive oil were added to minimize foaming, served as the collecting fluid. Its volume, accurate to about 0.1 ml, was estimated from the density of the fluid (1.005) and the weight of the flask empty and after sampling. After counting the colonies on pour plates made with appropriate aliquots (0.5 to 2.0 cc) and incubated 48 hours, the concentration of viable organisms, collected in each flask, per L of aerosol was estimated as follows:

$$\frac{\text{Volume collecting fluid (ml)}}{\text{Volume aliquot (ml)}} \times \frac{\text{Number of organisms in sample}}{\text{Sampling rate (1/min.)} \times \text{duration (min.)}}$$

= number of viable organisms/L aerosol.

When samplers were used in series, the total concentration of viable organisms was calculated according to the following considerations. It can be shown that as a first approximation $1-(N_2/N_1)$ = collection efficiency (C. E.) where N_1 and N_2 denote the number of viable organisms/L of aerosol collected by the first and second bubblers respectively, when they are in series. The calculated concentration is then: $(1/\text{C. E.}) \times N_1$.

Certain assumptions are, however, implicit in this calculation: (1) that the two collectors are structurally and dimensionally alike, especially with respect to orifice diameter and the distance between the orifice and impinging surface; (2) that organisms present in the aerosol are singlets; (3) that jet velocities are identical in both bubblers; and (4) that organisms are not destroyed, nor are their size and density changed during collection.

The first assumption is apparently fulfilled, since critical pressure flow rates of the collectors used in later experiments differ by less than 4% (0.3 L at 9.0 L per minute), and the jet is situated 5 mm from the bottom of the flask. Our earlier results suggest that the second is justified with *Bacillus globigii* spores, and for *Serratia marcescens* (Ferry et al, 1949). It would also appear applicable to *Esch. coli*, which exist as single organisms in the suspensions used by us. In the case of *M. candidus*, microscopic observation of samples impinged on glass slides accords with this assumption as do the later experimental data subsequently considered below.

The third assumption is certainly not exact. One can easily show that the velocity of air

issuing from the second jet is about 20% greater than that from the first at a flow of either 6.0, 4.5 or 3 L per minute.* On this basis, a second approximation, based on a correction linear in the velocity is possible. Such a correction appears itself to be an approximation especially applicable to collection efficiencies within the range 25 to 75%, and not beyond this range. Since linearity in the velocity cannot be assumed (May, 1945), and since application of the correction appears to change the value for calculated viable concentration by less than 5% at flow rates of either 6.0 or 4.5 L per minute, it has not been employed here.

The fourth assumption will be considered later under the heading DISCUSSION.

RESULTS

In table 1 appear typical results obtained from the study of freshly generated aerosols containing *M. candidus* and *Esch. coli*. The aerosols, generated at 25 C and not over one second old, were sampled over 4-minute periods. Sterility controls of filtered air were sampled for two minutes. Although the table is nearly self-explanatory, it should be noted that the differences in the number of colonies grown from different aliquots taken from the first sampler accord acceptably with those expected on statistical grounds, and that the standard deviation of the mean for the first sample is, presumably, about 5% of the count in the case of *M. candidus*, and about 7% in the case of *Esch. coli*. Although the percentile deviation is much greater for the second sampler, the numerical error is still small.

Cursory examination of the data reveals striking differences between the two organisms. With *Esch. coli*, photoelectronic particulate count rises about 30% within 52 minutes, in contrast with an initial 10% increase observed at the end of 41 minutes in the case of *M. candidus*. Moreover, the collection of viable organisms appears to be more effective in the case of the latter organism. In addition, increase in apparent viability of the suspension during an

TABLE 1.—*Collection of viable bacteria from aerosols.*

Run		Elapsed time in min.	Vol. of fluid (ml)	Flow rate L/min.	Number of viable colonies: from 1 ml					Number of viable colonies: from 2 ml					Mean no. of organisms: per ml fluid	Mean no. of organisms: per L aerosol	Particulate concentration, no./L: Viable calc.	Particulate concentration, no./L: Photoelectronic
								*M. candidus**										
Blank	A				0	0	0			0	0	0						
I	A	25	25.2	6.0	290	265	290	306	306	566	623	564	623	525	290	304	334	890
	B		24.1	6.0	26	30	32	25	23	51	46	69	54	47	27	27		
II	A	34	24.3	4.5	206	221	X	202	242	427	428	413	431	394	214	289	340	922
	B		25.0	4.5	20	33	31	42	30	64	51	62	57	71	31	43		
III	A	41	24.8	3.0	96	85	75	81	93	160	172	180	154	161	85	176	310	980
	B		26.2	3.0	39	40	30	42	38	47	56	58	78	78	35	76		
								Esch. coli†										
Blank	A				0	0	0			0	0	0						
I	A	13	24.7	6.0	108	94	117	121	98						108	111	116	886
	B		24.6							5	7	8	6	9	4	4		
II	A	63	24.6	6.0	80	89	80	90	70						82	84	88	1356
	B		24.7							10	4	4	9	4	3	3		
III	A	122	24.8	6.0	59	81	76	55	82						71	73	76	1708
	B		24.6							4	5	4	4	4	2	2		

A and B refer to first and second collectors, respectively.
* Viability of suspension %, initial 24, final 28. Relative humidity of aerosol 64%, temperature 25 C.
† Viability of suspension %, initial 60, final 49. Relative humidity of aerosol 70%, temperature 25 C.

* We are not aware of any method by which it is possible for us to obtain critical pressure flow in both of two impingers in series.

experiment contrasts with the decrease observed in similar studies with *Esch. coli*. These observations suggest that the former organism is more rugged and persistent than the latter, which rapidly loses viability in the suspension atomized in a refluxing atomizer, as well as in the aerosol generated therefrom.

Figures 1a and b contrast the change of particulate count represented by the ordinate observed in aerosols of *M. candidus* and of *Esch. coli* during the course of a run in which elapsed time in minutes is given as abscissa.

In figure 1a, results obtained with *M. candidus* are represented graphically. Here the diagonal crosses indicate the concentration was attained when either formalinized suspensions of *M. candidus* or *Esch. coli*, or living suspensions of the former were atomized, this did not occur with living suspensions of the latter. When such suspensions were atomized, particulate concentration increased continuously reaching 170% of its initial value within two hours. It is noteworthy that although constant concentration was rapidly reached with dead *Esch. coli*, it was observed only after *M. candidus* had been atomized for about 30 to 40 minutes.

Although it is possible qualitatively to interpret the differences between the two organisms as suggested by table 1

TABLE 2.—*Decrease in viability of* Esch. coli *suspensions during atomization at 25 C.*

Date	12/17/48	1/21/49	1/25	2/1	2/25	3/3	3/8	3/18	3/22	5/5	5/6	6/16	6/22
% viable organisms													
Initial	79	88	(86)	77	73	48	67	75	(72)	58	60	41	40
Final	63	53	56	54	61	23	32	26	26	43	49	15	16
Relative viability at end of experiment	0.80	0.60	0.65	0.70	0.83	0.48	0.48	0.35	0.37	0.74	0.82	0.36	0.39
Duration of experiment in min.	40	70	65	54	75	107	135	123	134	96	122	119	125
Age of suspension in day	14	3	6	13	2	8	13	2	6	1	2	1	1
$-k$	2.4	3.2	2.9	2.9	1.1	3.0	2.4	3.7	3.3	1.4	0.7	3.7	3.3
Number of fragments assumed	3	2		2	2 to 3	2 to 3	3 to 2	3	2 to 3	2 to 3	2 to 3	2	2

actual counts obtained during the atomization of a living suspension, the circles represent points obtained with a killed suspension. But since these aerosols differed in concentration, values observed with the killed suspension have been multiplied by an arbitrary factor of 1.5 in order to facilitate visual comparison.

In figure 1b, the diagonal crosses indicate observations made on an aerosol obtained from a live suspension, the circles relate to an aerosol generated from a similar suspension killed with 0.5% formalin. The curves shown are calculated on the basis of considerations subsequently discussed.

Inspection of these figures reveals differences related to the organisms used. Although nearly constant particulate and figure 1, quantitative interpretation must rest on data presented in condensed form in tables 2 and 3.

The former illustrates the decrease in viability at 25 C of suspensions of *Esch. coli* as they repeatedly pass through the atomizer orifice. The initial percentage of viable organisms is that of the suspension introduced into the atomizer; the final percentage denotes that of the residual suspension at the end of an experiment. The values given relate to all suspensions studied that were not over two weeks old. Two values for initial viability in parenthesis were estimated from an observed daily loss of viability approximating 1% during the first few days of standing. All other values were directly measured as described under

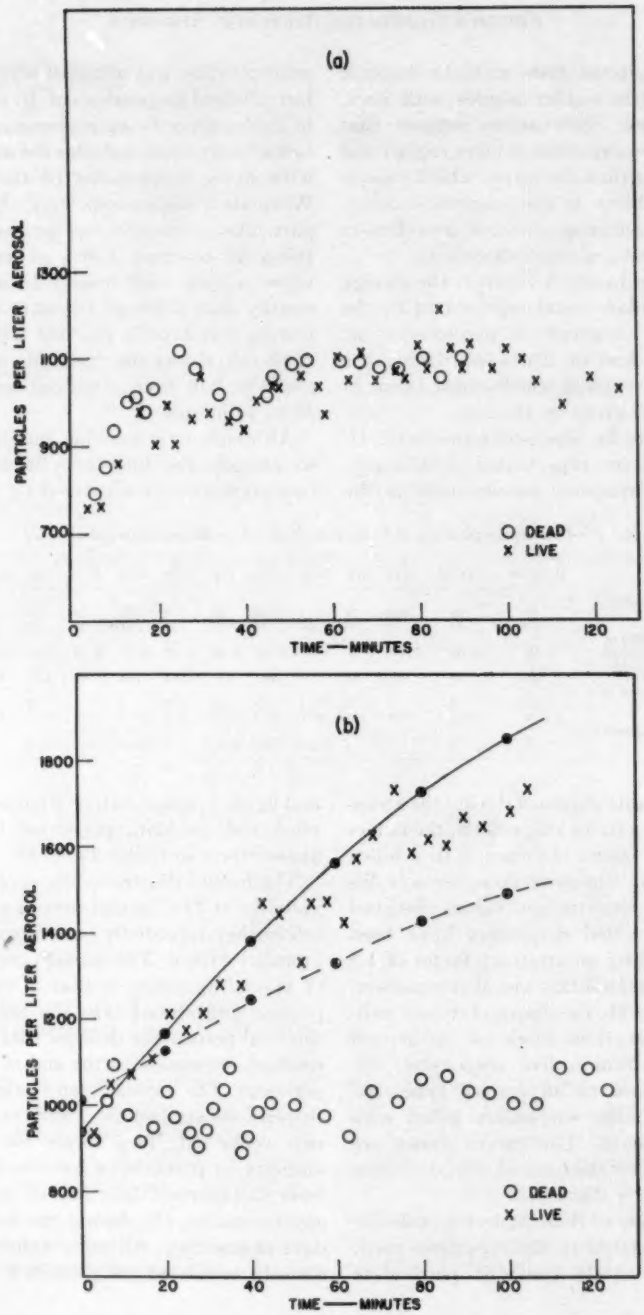

FIG. 1.—Particulate concentration of aerosols during atomization. a. *M. candidus;* b. *Esch. coli.*

METHODS. Relative viability at the end of experiment denotes the value of the ratio of final viability:initial viability and appears in the 4th row of the table. The negative logarithm of this number divided by the duration of the experiment in minutes and multiplied by 10^3 appears as $-k$ and characterizes the

TABLE 3.—*The viability of freshly generated bacterial aerosols.*

Date	Suspension		Elapsed time in min.	Biological sampling rate L/min.	Particulate concentration of aerosol no./L				Collection of viable organisms	
					Viable organisms		Total	Viable expected	Apparent efficiency %	% Total particles
	Age in days	Viability %			Total	1st Collector				
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
					M. candidus					
2/11/49	1	37*	19	4.5	288	218	738	273	105	39
			30	3.0	247	160	772	286	86	32
			39	6.0	302	274	808	299	101	37
2/15/49	5	24(37)	25	6.0	334	304	890	249(330)	134(101)	38
			34	4.5	340	289	922	258(342)	132(99)	37
			41	3.0	310	176	980	276(362)	112(86)	32
2/23/49	13	26	97	9.1		292	1074	280	109	27
			104	9.2		291	1094	284	103	27
			113	3.0	181	150	988	258	70	18‡
			120	6.0	248	236	1054	274	91	24‡
			126	6.0	237	228	1024	266	89	23‡
					Esch. coli					
2/25/49	1		0				750			
		53(65)†	46	9.0	111		1096	399(485)	28(23)	10.1
		50(63)	56	9.1	120		1178	375(472)	32(25)	10.2
		48(62)	62	6.0	148	136	1356	362(465)	41(32)	10.9
		46(61)	69	6.0	146	139	1320	345(460)	42(32)	11.0
3/3/49	7		0				900			
		46	7	6.0	76	73	1068	413	18	7.1
		34	52	9.2	89		1328	304	29	6.7
		32	58	6.0	78	76	1468	292	27	5.3
		31	65	9.0	74		1568	268	26	4.7
		25	101	6.0	76	73	1680	221	33	4.5
3/8/49	12		0				900			
		62	12	7.0	84	81	920	554	15	9.1
		46	57	9.0	70		1395	412	17	5.0
		44	63	6.0	63	61	1390	394	16	4.5
		42	70	9.0	45		1385	379	12	3.2
		34	102	6.0	54	49	1650	306	18	3.3
3/18/49	1		0				900			
		67	15	6.0	85	77	1010	600	14	8.4
		58	36	6.0	92	82	1540	524	18	6.0
		43	82	6.0	62	52	1200	384	16	3.0
			123							
3/22/49	5		0				900			
		66(34)	13	6.0	86	79	1062	600(306)	15(29)	8.1
		57(32)	35	6.0	69	62	1254	514(288)	13(24)	5.5
		43(29)	78	6.0	73	63	1536	384(262)	19(28)	4.8
		31(26)	128	6.0	65	57	2020	308(234)	21(28)	4.2
5/5/49§	1		0				1350			
		56	13	6.0	218	207	1328	758	29	15.6
		54	33	6.0	152	149	1646	720	21	9.2
		47	92	6.0	157	154	1988	620	25	7.9
5/6/49§	2						825			
		58	13	6.0	116	111	886	478	24	13.1
		52	63	6.0	88	84	1356	423	21	6.5
		54	122	6.0	76	73	1708	355	21	4.5
6/22/49	1		0				650			
		36	13	6.0	53	51	750	236	23	7.1
		32	33	6.0	33	31	846	206	16	3.9
		26	63	6.0	35	33	799	169	21	4.4
		18	121	6.0	24	23	1009	114	21	2.8

Experimental conditions: temperature 25 C, relative humidity, runs prior to 3/18, 65±3%, 72±2%.
* Values for viability of *M. candidus* are observed values.
† Values for viability of *Esch. coli* calculated by methods described in text.
‡ No olive oil.
§ $k = -0.001$.

rate of decay of the suspension.

The values for k appearing in the table show reasonable consistency except for three strikingly low values. Although some correlation between decay rate and the age of the suspension might reasonably be expected, it is not suggested by the present data. None the less, two of the low values refer to a suspension used in May, which was prepared more rapidly than those employed earlier. It is on that account that we have omitted these values in deriving the mean, 2.9×10^{-3}. The aberrant value for the experiment of 2/25, although not directly explicable, is not completely unexpected on statistical grounds.

These data are graphically summarized in figure 2, in which the logarithm of relative viability as ordinate is plotted against duration of run in minutes as abscissa. It appears that a line drawn with a slope of -0.0029 fits the data satisfactorily. None the less, it seems possible that even under the uniform experimental conditions imposed, individual preparations have characteristic decay constants differing slightly from each other. Since, however, determination of relative viability is rather inexact, largely because of variation in direct bacterial counts, significant individual differences in the constant are not demonstrable.

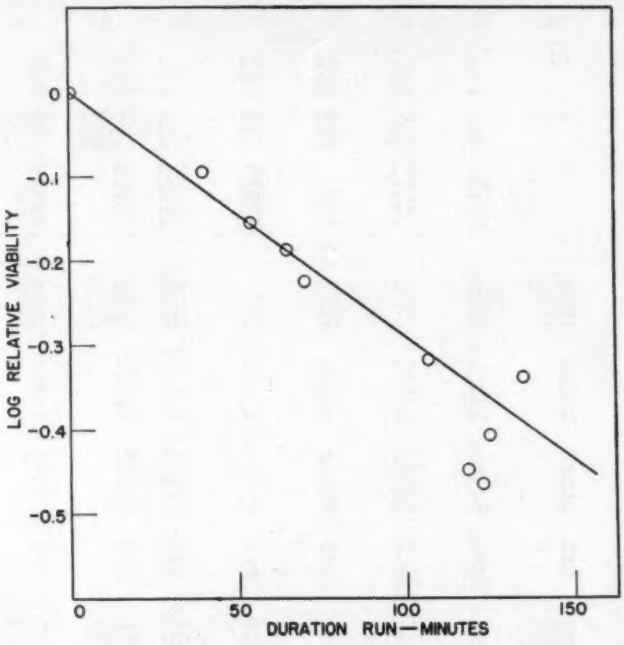

FIG. 2.—Decreasing viability of *Esch. coli* suspension during atomization.

Use of a modified atomizer in which fluid enters the delivery jet from an outside reservoir suggests that the suspension passes through the jet at a rate of about 30 ml per minute. This implies that 10 ml of suspension ordinarily introduced are atomized initially at least 180 times an hour and more frequently

as its volume decreases. Loss in viability is, therefore, about 0.2% following a single passage through the orifice. But, even under the rather uniform conditions employed here, it does not seem unlikely that different preparations may have different decay constants.

We can take a further step in analyzing the data in table 2. The observed increase in particulate concentration accompanied by diminution in the number of viable organisms is readily explicable if we assume that as organisms break, viability of the suspension decreases. Using the mean decay constant, -0.0029, it is possible to estimate viability during the course of an experiment by means of the equation

$$\log \text{viability} = \log (1-x) = kt \qquad (1)$$

where x = proportion of organisms destroyed, t, time in minutes. We can also assume that each organism breaks alternatively into 2, 3, or 4 major fragments, all detectable by the counter, and readily compute the particulate concentration corresponding to each assumption. As the curves in figure 1b suggest, the data are best fitted by a number of fragments between two and three. This assumption is further supported by the numbers in the last row of table 2 which represent the number of major fragments necessary to fit the time-concentration curves for each preparation. This number also lies between two and three.

We next turn to table 3, in which experimental data for *M. candidus* and *Esch. coli* are presented in condensed form. All experiments were performed at 25 C, and at relative humidities between 62% and 74%. Omitted are the results of preliminary experiments which suggested that with *Esch. coli*, a single collector was most effective at a flow rate of 6.0 L per minute, and that at greater or lesser flow rates collection was less efficient. We have, however, included the results of all experiments in which two all-glass collectors were used in series, as well as some data for collection at sonic velocity. Since the table is nearly self-explanatory, few comments are necessary. In table 3, column 3, percent viability of suspension represents observed viability at the end of a run; with *Esch. coli*, the values given are calculated according to the method already described.

Values appearing under "Collection of viable organisms Apparent efficiency %" represent

$$\frac{\text{Total viable concentration}}{\text{Expected viable concentration}} \times 100.$$

Those in the last column headed "% Total particles" indicate

$$\frac{\text{Total viable concentration}}{\text{Total particulate concentration}} \times 100.$$

M. candidus.—The data referring to *M. candidus* are easier to interpret and will, therefore, be considered first. An initial gradual rise in particulate concentration followed by prolonged constant concentration of an aerosol generated from living or dead suspensions has been repeatedly observed and may be correlated with the breakage of many clumps in the suspension atomizer. This has been independently established by direct differential estimation of the proportion of bacteria in the suspension occurring either singly, in doublets, or in clumps of three or more, both at the beginning and at the end of an experiment. At the end, singlets predominate. The fact that time-particulate concentration curves follow the same course with living or dead organisms suggests that few, if any, cells are destroyed during atomization.

Although the determination of viability in clumped suspensions is undoubtedly uncertain not only because

direct counts are difficult and probably inaccurate, but also because the number of colonies does not accurately reflect the number of viable organisms (Ferry et al, 1949), our data do not suggest that repeated passage through the refluxing nebulizer decreases viability. Although it has been shown that all of the suspension initially passes through the nozzle about 180 times an hour and more frequently as its volume decreases, we have never noted any significant decrease in viability. We have, however, observed large increases on two occasions. None the less, percentile viability must always be cautiously accepted. With *M. candidus*, the terminal determination is, we believe, more reliable, since clumps are few in number.

Collection of viable organisms from aerosols also suggests that *M. candidus* is very resistant to the mechanical trauma to which it is subjected during atomization and collection. Under the experimental conditions, the collection efficiency of a single collector is shown to increase with jet velocity. In preliminary experiments where collection was made at critical pressure flow, we had noted extraordinarily high efficiencies, presumably due to low estimates of viability in badly clumped suspensions. These early observations suggested that *M. candidus* resists rather severe mechanical shock. This interpretation is supported by later experiments with fresher material, in which the ratio of the concentration of viable organisms collected to the total particulate concentration observed approximates the apparent viability of the suspension atomized.

A few values included in the data for 2/15/49 appear in parentheses. Since we regularly had noted little or no deterioration in *M. candidus* suspensions during a week, the observed value for final viability, 24%, seemed unduly low in comparison with that noted in the preceding experiment. Accordingly, we tentatively assumed the value 35% observed 5 days earlier and calculated expected viable concentration and apparent collection of viable organisms as before. The results so obtained appear to yield more consistent values for collection of viable organisms.

Although the results of 2/23/49 qualitatively accord with those from other runs, they also serve to illustrate the magnitude of losses by entrainment when a suitable antifoaming agent is omitted.

Consideration of all the data confirms the impression that with this organism, the apparent concentration of viable particles is independent of the jet velocity at which collection is made (Ferry et al, 1949). Since clumps, if present, might be expected to break more completely at higher velocities, thereby giving rise to higher viable counts, it seems reasonable to conclude that the particles counted are predominantly single organisms. The data strongly suggest, moreover, that *M. candidus* is indeed resistant to mechanical shock. Although concentrations calculated from collections made at different flow rates are in fair agreement when two collectors are used in series, a flow rate of 6.0 L per minute appear to be most satisfactory, since a large proportion of the organisms collected appear in the first collector. Consequently, the relatively large, though numerically small, variation in the number of bacteria appearing in the second sampler leads to insignificant differences in the estimate of total concentration.

For purposes of comparison, jet velocities computed from orifice diameter, pressure drop across orifice and flow rates are indicated in table 4.

Esch. coli.—Data relating to *Esch. coli* are assembled in table 3. Since via-

TABLE 4.—*Jet velocities.*

Flow rate L/min.	Jet velocities Meters per second	
	Sampler	
	1	2
9.1	348	
6.0	148	186
4.5	103	109
3.0	67	71

bility of the suspension decreased during the course of an experiment, it was necessary to use values calculated by means of equation 1, corresponding to the time at which samples were taken. Except for the suspensions studied in May, the constant used has generally been taken as $k=0.0029$. For the experiments of May 5 and 6, a value of -0.001 has been taken. Other tabulated values are derived by the methods already indicated.

In the case of two experiments, those of 2/25 and 3/22, second sets of values appear in parentheses. Those relating to the former are calculated by means of the constant $k=-0.0011$ derived directly from this experiment; those relating to the experiment of 3/22 are derived from a constant based on observed initial and final values for viability of the suspension. The fact that apparent recovery based on these calculations accords better with that found in other experiments tends further to support the hypothesis that decay constants are in fact characteristic of each preparation studied.

The data obtained with aqueous suspensions and aerosols of *Esch. coli* differ significantly from the experimental data with similar preparations of *M. candidus*. In the first place, suspension viability decreases as atomization is continued. Second, the particulate count rises continuously during a two-hour to two-and-a-half-hour experimental period. Third, a collection of viable organisms from aerosols at sonic jet velocity frequently seemed to be less efficient than that at slower flow rates; fourth, the proportion of viable organisms collected and cultured is much lower than that expected from the suspension viability; fifth, the ratio of the concentration of viable to total particles in the aerosols decreased rapidly even at a constant collection rate, never exceeding 16% in the experiments reported here.

Although decrease in viability of the suspension can be quantitatively accounted for in terms of breakage during atomization, the recovery of viable organisms from resulting aerosols seldom exceeds about 30% of the values expected. With fresh preparations, a mean recovery about 25% of that expected was observed, and this despite the fact that the larger organism should be more readily collected.

DISCUSSION

The contrast between the behavior of *M. candidus* and *Esch. coli* is indeed striking. Although the former organism apparently withstands atomization and collection, the latter surely does not. Fracture during atomization, though demonstrable, is insufficient to account for the low recovery of about 25% of the viable *Esch. coli* present in aerosols.

None the less, it is possible to ascribe this phenomenon either to extensive breakage during collection or to death during drying. The latter might be expected to be less important in aerosols less than one second old. Breakage within the atomizer might well be less, since the organisms in the atomizer are generally enclosed in water, which probably protects them, partly because of its high surface tension. In the aerosol, the particles are nearly dry, so that the impact on collection at relatively high velocity makes fracture plausible.

Although the experimental conditions are not such that bacterial killing by

rapid changes in osmotic and chemical activity within and without the cell cannot be definitely excluded, differences in the cellular structure of *M. candidus* and *Esch. coli* revealed by phase contrast microscopy, together with the electronic microscope studies of the latter by Hillier et al (1949) suggest that breakage may be an important factor.

These observations show that *M. candidus* occurs as nearly spherical particles with a high and uniform optical density. *Esch. coli*, in the 12- to 16-hour-old cultures used here, consists of rods in which one generally observes two or three optically dense foci separated by attenuated areas. Whereas the shape and structure of the former organism are consistent with resistance to mechanical shock, the presence of two or three dense regions separated by a much less dense structure suggests that the two to three fragments calculated from the data and given in table 2 are related to breakage of the structure of the latter organism. It is, moreover, well known that bacteria differ in resistance to ultrasonic desintegration, and in particular Stumpf et al (1946) have shown that *Esch. coli* is sensitive and *Micrococcus lysodeikticus*, an organism presumably similar to *M. candidus*, is resistant to this treatment.

Although it is, therefore, probable that shape and structure affect resistance to mechanical shock and consequently the survival and collection of bacteria under the experimental conditions employed, further investigation is necessary to exclude concomitant lethal effects of changes in chemical environment during the drying process. A projected study comparing collection by impingement with filtration under suitable conditions should help differentiate between these effects.

It seems possible that form and structure should be included as factors influencing the persistence and infectivity of organisms causing respiratory infection. Although air velocity during atomization as described here presumably exceeds 100 m per second, similar velocities may be realized during coughing and sneezing. Jennison (1942) has indeed observed maximal particulate velocities of 46 m per second, at a distance of two inches from the mouth and estimates considerably higher velocities in coughing. He also recalls earlier experiments of Chaussé and Magne (1916) who concluded that while comparable velocities were attained in the unconstricted glottis, materially higher velocities were possible during constriction. Since small particles rapidly decelerate when still air is encountered, and since constriction of the larynx undoubtedly occurs during coughing, high initial velocities necessary for atomization appear possible. These, however, would probably account for the destruction of a very small proportion of the organisms expelled.

Many of these organisms occur in larger droplets so that the contained bacteria are heavily coated and settle rapidly. Some may be nearly naked and settle slowly. Although they undoubtedly will not encounter the high collection velocities experimentally attained, more fragile organisms should be more readily destroyed. Thus, form and structure may be factors affecting the persistence of airborne organisms, and consequently respiratory infectivity. Further information of the characteristics of a variety of organisms is, however, necessary to assess the importance of this factor.

SUMMARY AND CONCLUSIONS

1. Freshly generated aerosols of *Escherichia coli* and *Micrococcus candidus* have been studied simultaneously

by means of photoelectronic and biological counts.

2. The methods used are briefly described.

3. *M. candidus* and *Esch. coli* differ markedly in their behavior both in the aqueous suspension atomized and the resulting aerosol.

4. The former organism is resistant to mechanical shock and appears to have approximately the same viability in an aerosol, as in the suspension used for generation.

5. Although *Esch. coli* in suspension are gradually destroyed during atomization, this is insufficient to account for observed recovery from aerosols, which may be tentatively ascribed either to killing during drying or fracture during collection.

6. A quantitative description of the differences between the two organisms has been attempted in terms of their form and structure.

7. It is suggested that the form and structure of organisms may be related to their persistence in air.

8. Although form and structure may be factors affecting the persistence and infectivity of airborne organisms, further study of a variety of organisms is necessary to assess the importance of this hypothesis.

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APPENDIX

Although the photoelectronic counter described by Gucker et al (1947) appeared to measure particulate concentration of aerosols provided the effective diameter of particles somewhat exceeded 0.7μ, refinements were necessary to insure satisfactory counts of smaller particles. In addition, some changes in the aerosol generating system were necessary in order to provide aerosols reasonably constant in composition. And even if further improvements in the photoelectronic system now seem both possible and likely, it seems desirable to describe the changes already made in experimental procedure not only as a guide to further improvements, but also because the results related here can then be better compared with results obtained with even better apparatus. For convenience, we have collected the changes under two headings: *Generating system* and *Counter*.

Generating system

The changes in this system, though slight, are significant. In order to compensate for evaporative loss of water and consequent concentration of the bacterial suspension and the aerosol, we have added water to the suspension at an appropriate rate. This was accomplished by means of a motor driven syringe which could deliver water into the suspension at any desired rate from about 0.01, to more than 0.1 cc per minute. The actual delivery rate was about 0.04 cc per minute. With *M. candidus*, after an initial period in which clumps were broken, an aerosol

of nearly constant concentration was produced. This was illustrated in figure 1a.

In addition, the atomizer used, although of the type previously described, employed the side of the generating vessel, situated about 5 mm from the air and liquid orifices, as a baffle. The particulate output per minute is considerably less than that observed with the earlier form at comparable rates of delivery when this is measured in terms of air flow and weight of water lost. This suggests that only the smaller droplets and particles escape and enter the final aerosol. The atomizer is illustrated in figure 3. We still believe that for the present, it is desirable to use carefully washed aqueous bacterial suspensions, since only under these conditions is particulate size in the aerosol determined by that of the bacteria, thus aiding in the generation of nearly uniform aerosols so essential for careful studies. It should be noted that although our earlier studies suggest that most of the particles counted consist of single bacteria, a few doublets may still be present.

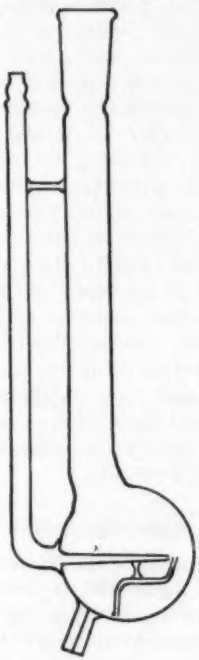

FIG. 3.—Atomizer.

Counter

The modified counter cell is shown in figure 4. The changes in design are numbered in this figure, and are as follows:

Optical system.—Back of the tungsten headlight bulb is a concave spherical mirror (1), so placed and with a radius such that backward radiated light is redirected toward the filament, thereby markedly increasing the light intensity at the focus within the cell.

The glass plate used to seal the cell has been eliminated by using one of the condensing lenses for this purpose. To the other is cemented a spherical black glass mirror backed by a sheet of tin foil. The mirror which has its center of curvature near the center of the collecting lens (7) absorbs and redirects stray light within the cell; the foil eliminates transmission of red radiation.

The bakelite blocks inserted in the cell cut down cell volume.

The tube 6, which extends toward the photocell, was redesigned and now contains a triple aplanat lens (7) coated to diminish reflection. It is so placed that only a small volume about the focus of the condensing lenses can be viewed; thereby decreasing background illumination of the phototube.

A smaller circular stop (8) further reduces background illumination.

A filter holder (2) is provided so that monochromatic light can, if desired, be used.

Electrical system. Photocell.—The

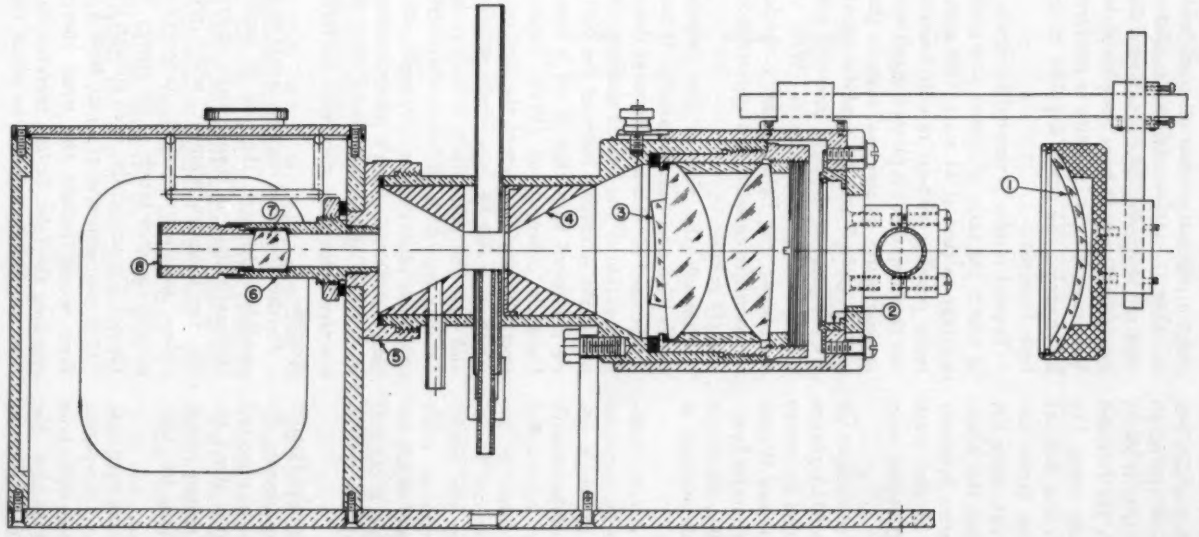

FIG. 4.—Modified photoelectronic counter.

use of a 931A photomultiplier selected for sensitivity, has theoretical advantages. First, the tube has a high frequency response, and second, the maximum of its spectral response is at about 4000 Å as compared with the infrared maximum of the thalofide tube. For particles having a diameter less than the wave length of light, this latter advantage might well be great, since the Raleigh theory implies that the intensity of scattered light varies inversely with the fourth power of the wave length. For objects of bacterial size, this advantage is less.

A well-regulated power supply is essential for use with the 931A phototube. This has been provided by means of a circuit devised by Professor Wayne B. Nottingham, of Massachusetts Institute of Technology. At 800 v, the circuit showed a measured ripple voltage of about 0.005 v or 0.006%.

A scale of 64 scaling circuit, without moving parts, markedly increases the maximum frequency at which impulses can be counted as compared with that observed with a mechanical counter. Its use does not, however, imply that the counting rate can be indefinitely increased, since the simultaneous appearance of two or more particles in the field of view which becomes increasingly probable as concentration increases, also imposes restrictions.

When *M. candidus* is atomized under the experimental conditions already described (Ferry et al, 1949) the observed S/N (signal to noise ratio) is about 10. The sensitivity of the system used, therefore, appears adequate for bacterial counting.

The completeness of count may conveniently be checked by comparing counts at low light intensities with those at higher intensities. Provided the aerosol is reasonably homogeneous, counts should initially increase rapidly with increasing light intensity, and reach a maximum when all particles in the field are sufficiently illuminated so that the scattered light produces a distinguishable signal from the phototube. Variation of light intensity is produced by changing the power supplied to the light filament.

Typical results presented in figure 5 in which the ratio of counts at a given wattage to that at 41 watts (the maximum possible without rapidly burning out the lamp used) is plotted as ordinate against watts as abscissa, suggest that with *B. globigii* and *M. candidus* counts are complete at the light intensity provided by about 30 watts. With *S. marcescens*, the light intensity needed for complete enumeration appears to be slightly greater.

Although the size of the impulse varies with particulate diameter, it cannot be used for this measurement unless illumination of the field is uniform and unless the refractive index of the particle compared with that of air is fixed. Gucker's observations (1949) suggest that with our instrument the first condition may not be completely satisfied and that the particulate refractive index may vary with the relative humidity at which observations are made. Our own observations with a phase-contrast microscope suggest, moreover, that refractive index may vary with bacterial species.

Further technical improvements seem possible. Although the use of monochromatic light is theoretically desirable, the tungsten filament, which provides satisfactory and convenient source, radiates much of its energy in the red. Consequently, even though the 931 is most sensitive at about 4000 Å, a filter would reduce the total energy reaching the tube. It has, therefore not been employed. In order to avoid this

difficulty, the use of a mercury arc was considered but seemed to be impractical since D C operation, though possible, entails elaborate voltage regulation. Without the use of appropriately regulated circuits, variations of light intensity would certainly give rise to spurious impulses.

Gucker (1949) has recently perfected a cell in which 90° scattering is scanned. Although background illumination and noise is reduced to an extremely low level, the observed S/N ratio with *B. globigii* did not appear much greater than that observed by us.

It does, however, seem likely that improvements in the optical and electrical systems will decrease the minimal size at which accurate counts are possible. By the use of selected 931 or 1P21 tubes, sensitivity can be increased. Increase in sensitivity will, however, necessitate careful control of background illumination. Since gain in the 931A photomultiplier is great, the rest of the amplifying system can be operated at lower gain and, therefore, under more stable conditions.

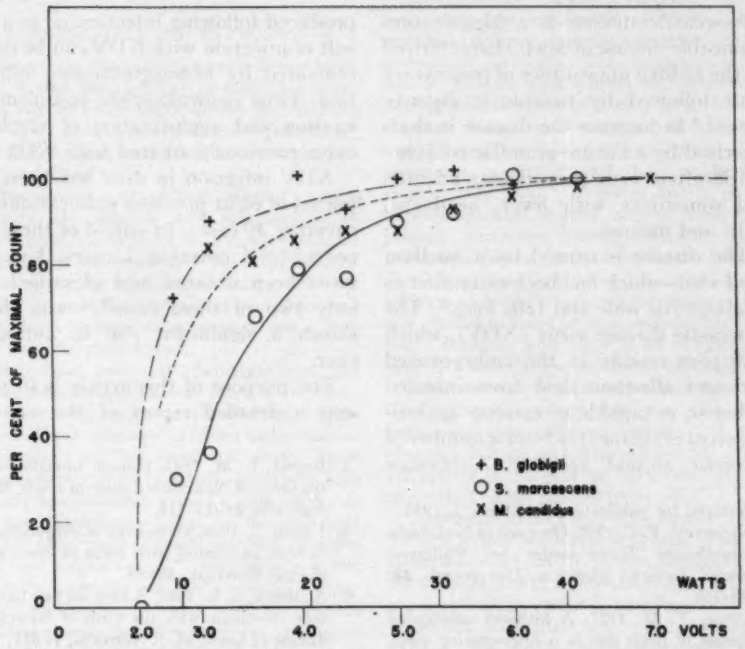

FIG. 5.—Light intensity and particulate count. Intensity of illumination is a function of wattage which appears as abscissa, particulate concentration as ordinate. Particulate concentration appears to approach a limiting value asymptotically. This is reached at approximately 31 watts with the organisms studied.

LABORATORY ASPECTS OF AN INFECTION WITH NEWCASTLE DISEASE VIRUS IN MAN

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Newcastle disease is a highly communicable disease of fowls characterized by the sudden appearance of respiratory signs followed by neurologic disturbances.[1,2] In humans the disease is characterized by an acute granular conjunctivitis often with preauricular adenitis and sometimes with fever, headache, chills and malaise.

The disease is caused by a medium sized virus which has been estimated to be about 70μ wide and 180μ long.[3,4] The Newcastle disease virus (NDV), which multiplies readily in the embryonated egg[5] and allantoic fluid from infected embryos, is capable of causing agglutination of erythrocytes from a number of different animal species.[6] Antibodies produced following infection or as a result of injection with NDV can be demonstrated by hemagglutination inhibition, virus neutralization, complement fixation and agglutination of erythrocytes previously treated with NDV.

NDV infection in man has been reported in eight previous publications[7–14] covering 39 cases. In only 4 of these reports,[7,9,12,13] covering 7 cases, has the virus been isolated and identified. In only two of these cases[7,12] was there shown a significant rise in antibody titer.

The purpose of this article is to present a detailed report of the various

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laboratory aspects of NDV infection in the human, not complicated by possible concurrent bacterial infection and to record certain new findings discovered in the case observed. The clinical aspects of the infection from which these studies were made are reported elsewhere.[15]

MATERIALS AND METHODS

Virus.—The strain of Newcastle disease virus used in the hemagglutination-inhibition titrations was the strain which caused the infection. This strain was originally isolated by Beaudette[16] and was obtained from The Children's Hospital of Philadelphia.

This virus sample had been cultured in chick embryos, the allantoic fluid harvested in 48 hours, pooled, distributed in a number of small vaccine vials and frozen at −20 C. The material which caused the infection was from one of these vials. All hemagglutination-inhibition titers were determined by using virus material from a fresh vial from the original stock.

Red blood cells.—All hemagglutinin and hemagglutination-inhibition titers were determined by using human type O cells from a single source. Fresh cells were collected and preserved in Alsever's solution every two weeks. These cells gave reproducible results throughout such periods when checked with the stock virus suspension. Immediately before use, the cells were washed three times in sterile physiological saline and then prepared as a 2% suspension in saline. In both hemagglutinin and hemagglutination-inhibition titration, cells were added to either the virus suspension or the serum-virus mixture to make a final cell concentration of 0.66%.

Hemagglutinin titration.—The materials to be tested for hemagglutination capacity were diluted in saline in twofold serial dilutions from 1:10 to 1:10,240. To 0.5 ml of each of these dilutions was added 0.25 ml of a 2% suspension of red blood cells in saline. The tubes were shaken and allowed to stand at room temperature for two hours. Readings were taken at 45 minutes and 2 hours, but no significant difference was noted at these two time intervals. The hemagglutination titer was recorded as the reciprocal of the greatest dilution, before the addition of red blood cells, which produced maximum agglutination. No attempt was made to interpolate titers on the basis of partial agglutination. A virus control (virus plus red blood cells) and a saline control (saline plus red blood cells) were set up with each test.

Hemagglutination-inhibition titration.—All serums were heat inactivated at 56 C for 30 minutes in order to minimize nonspecific hemagglutination inhibition. The hemagglutination titer of the stock virus was checked before each hemagglutination-inhibition test. Serial twofold dilutions of serum were made, and to 0.25 ml of each dilution was added 0.25 ml of the virus suspension containing two units of hemagglutinin. The "unit" of hemagglutinin is the minimum amount of virus material required to produce maximum hemagglutination. These tubes were shaken, and without further incubation 0.25 ml of a 2% red blood cell suspension was added to each tube. The tubes were again shaken and allowed to stand at room temperature for 2 hours. Readings were taken at 45 minutes and again at 2 hours, but no significant difference was noted in the readings at these two time intervals. The following controls were run with each hemagglutination-inhibition titration: (1) virus control (virus plus red cell suspension); (2) serum-cell control (serum plus red blood cells) and (3) cell control (saline plus red blood cells).

Complement fixation test.—The Beaudette and Victoria strains of Newcastle disease virus, the latter obtained from Dr. Burnet, were grown in the allantoic sacs of embryonated eggs. Infected allantoic fluid, untreated or centrifuged at 1500–2000 r.p.m. and dialized against phosphate buffer solution, was used as the complement fixation antigen. Multiple dilutions of the antigen and serums were employed. The technique of this test has been previously described.[17]

Neutralization test.—Serial dilutions of heat-inactivated serum were mixed with one or more dilutions of virus and allowed to stand for one and one-half hours at room temperature and for one-half hour at 4 C. In the experiment shown in table 6, serum dilutions were made as follows: undiluted, 1:25, 1:50, 1:100 and 1:500, and virus was used in dilutions of 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10}. The infective titer of the virus was 10^{-10} as defined by infection of chick embryos, demon-

15. Keeney, A. H. and Hunter, M. C. 1950, Human infection with the Newcastle virus of fowls. Arch. Ophth. **44**: 573–580.

16. Beaudette, F. R., Bivins, J. A., Miller, B. R., Hudson, G. B. and Black, J. J. 1949, Studies on the diagnosis of Newcastle disease in New Jersey. Am. J. Vet. Research, **9**: 69–76.

17. Sigel, M. M., Allen, E. G., Williams, D. J. and Girardi, A. J. 1949, Immunologic response of hamsters to influenza virus strains. Proc. Soc. Exper. Biol. & Med. **72**: 507–510.

strated by the presence of hemagglutinins. All inoculations were made via the allantoic route. Forty to 50 hours after inoculation, the eggs were chilled and allantoic fluids harvested. Presence of virus was determined by the agglutination of chicken erythrocytes following treatment of the fluids with sodium iodate. This procedure employed addition of 0.2 ml of *M*/10 sodium iodate to 1 ml of fluid, allowing the fluid to stand for 30 minutes at room temperature and adding 0.2 ml of 40% glucose. The purpose of this procedure was to eliminate rapid elution of the virus from red cells which may give "false" negative results.

RESULTS

On the second and fourth days after infection, washings were taken from both eyes and the nasal cavity. The washings were separately passed through Swinney filters containing Seitz disks (No. 423 FD) to remove bacteria. These filtered washings were then injected into the allantoic cavities of 12-day-old chick embryos. Each of three chick embryos was injected with 0.5 ml of washings from each source of each date. All of these chick embryos eventually hatched and there was no grossly discernible indication of infection. The failure to recover virus from these washings could be due to (1) free virus being absent from the washings in quantities sufficient to induce fatal infection, (2) adsorption of virus to the filter disk or (3) impermeability of the filter disk for particles the size of the NDV. Since the virus has been isolated earlier in the course of the human infection[7,9,12,13] and in the light of the work of Burnet[18] on the retention of NDV by Seitz pads, it is highly probable that virus was present in the eye washings, but the amount which passed through the Seitz filter pad was not sufficient to induce a fatal infection. Hemagglutination tests were not performed to rule out benign infection.

On the 6th day after infection, samples were taken from both eyes by first washing the outside of the eyelids with 70% alcohol and then taking eye washings in sterile physiological saline containing 500 units of penicillin per ml. Without any further treatment, these washings were injected into chick embryos. As can be seen in table 1, chick embryos injected with 0.6 ml, 0.8 ml and 1.0 ml of the washings from the left eye all died on either the third or fourth day after injection. The allantoic fluids of these chick embryos all showed positive hemagglutination titers of 1:320 or 1:640. Second passages were carried out by injecting 0.1 ml of allantoic fluid, yolk material and chick embryo tissue homogenate into additional chick embryos. All second passage embryos died on either the second or third day after being inoculated, and the allantoic fluids from these second passage chick embryos showed hemagglutination titers of 1:1280 or 1:2560. The allantoic fluids of all second passage chick embryos were injected into embryonated eggs in 0.1 ml quantities, and all of these third passage chick embryos died on the second day.

TABLE 1.—*NDV isolation in chick embryos from left eye on 6th day.*

Passage	Inoculum	Day of death	HT
1st	0.1 ml washing	4th	1:640
2nd	0.1 ml allantoic	3rd	1:1280
3rd	0.1 ml allantoic	2nd	
1st	0.8 ml washing	4th	1:320
2nd	0.1 ml allantoic	2nd	1:2560
3rd	0.1 ml allantoic	2nd	
1st	1.0 ml washing	3rd	1:640
2nd	0.1 ml allantoic	2nd	1:1280
3rd	0.1 ml allantoic	2nd	

HT = Hemagglutination titer.

A chick embryo injected with 1.0 ml of the right eye-washings of the 6th day eventually hatched. Another chick em-

18. Burnet, F. M., Beveridge, W. I. B., McEwan, J. and Boake, W. C. 1945, Studies on the Hirst haemagglutination reaction with influenza and Newcastle disease viruses. Australian J. Exper. Biol. & Med. Sc. 23: 177–192.

bryo which was injected with 1.1 ml of the same washings died on the 4th day, and the allantoic fluid of this chick embryo had a hemagglutination titer of 1:640. This allantoic fluid when injected into a second passage chick embryo in 0.1 ml quantity produced death on the third day. The hemagglutination titer of the allantoic fluid from this second passage chick embryo was 1:1280. One-tenth ml of allantoic fluid from the second passage chick embryo, when injected into the allantoic cavity, produced death of the third passage chick embryo on the second day (table 2).

Washings were again taken on the 18th day after infection by the same method as previously described and injected into chick embryos. Small samples of allantoic fluid removed 7 days after injection contained no hemagglutinin, and all chick embryos eventually hatched.

On the second and 5th days of infection scrapings were made from the lower cul-de-sacs, fixed with absolute ethanol and treated with Giemsa stain. Small granular inclusions varying in color from

TABLE 2.—*NDV isolation in chick embryos from right eye on 6th day.*

Passage	Inoculum		Day of death	HT
	ml	source		
1st	1.1	Washings	4th	1:640
2nd	0.1	Allantoic	3rd	1:1280
3rd	0.1	Allantoic	2nd	

HT = Hemagglutination titer.

burgundy to dark bluish-purple were demonstrated in the cytoplasm of the conjunctival epithelial cells. Scrapings made on the 17th day revealed entirely normal epithelial cells. Attempts to reproduce this cell picture in conjunctival scrapings from Newcastle virus inoculated rabbit eyes and in meningeal and chorioallantoic membrane scrapings from infected chick embryos were unsuccessful.

Samples of serum were collected for serologic studies on the 2nd, 4th, 11th, 14th, 18th, 43rd, 60th and 110th days after infection. The results of these serologic tests are discussed below.

Before heat inactivation of these serums, 0.1 ml quantities were injected into the allantoic cavities of 12 day-old-chick embryos. Serum taken on the second day, approximately 7 hours after a shaking chill which lasted about 30

TABLE 3.—*NDV isolation in chick embryos from serum of 2nd day.*

Passage	Inoculum	Day of death	HT
1st	0.1 ml serum	7th	1:160
2nd	0.1 ml allantoic	3rd	1:1280
3rd	0.1 ml allantoic	2nd	1:160

HT = Hemagglutination titer.

minutes, contained the virus as evidenced by serial passage through three chick embryos and positive hemagglutination in the allantoic fluids of each. As can be seen from table 3, this serum produced a unique reaction in the first passage in that the inoculated chick did not die until 7 days after inoculation and that there was very low hemagglutinating activity of the allantoic fluid. None of the serums obtained later than the second day following infection caused fatal infection of chick embryos.

The following three findings are considered of special importance: (1) the isolation of NDV from the blood stream has not previously been reported, (2) the systemic involvement in the patient was more severe than would have been expected from a mere local infection and (3) the transient nature of the viremia. The survival of the chick for 7 days following inoculation of serum may be indicative of a low concentration of virus in the serum, of partial inactivation of the virus by the so-called normal serum inhibitor or both of these factors.

Serologic studies.—The hemagglutination-inhibiting antibody of the patient's serum (table 4) was first detected

on the 6th day after infection. No further change was evident on the 11th day, but the titer rose to 1:128 on the 14th day and remained at that level on the 18th, 43rd, 60th and 110th days. A one tube decrease was noted on the 60th day after infection.

TABLE 4.—*Hemagglutination-inhibition titer of serum against NDV isolated from patient.*

Days after infection	Patient's serum	Control serum
2	1:4	1:4
4	1:4	1:4
6	1:16	1:4
11	1:16	1:4
14	1:128	1:4
18	1:128	1:4
43	1:128	1:4
60	1:128	1:4
110	1:128	1:4

In the complement fixation test (table 5) the Beaudette strain of NDV reacted strongly with the patient's serum. Some reaction was also observed with the Victoria strain. In the absence of a comparative test using Victoria antiserum and the Victoria virus, it is impossible to state whether the weaker reaction actually reflects antigenic dissimilarity between the strains or merely is the result of weak antigenic reactivity of the Victoria strain. We feel that the former premise is probably the true one.

TABLE 5.—*Complement fixation by patient's serum and Newcastle disease virus.*

Antigen	Antigen dilution	Serum titer
NDV (Beaudette)	Undiluted	1:32
	1:2	1:16
	1:4	1:16
	1:8	1:16
	1:16	1:8 (partial)
NDV (Victoria)	Undiluted	1:4 or 1:8
	1:2	<1:4 (partial)
	1:4	<1:4
	1:8	<1:4
	1:16	<1:4
Noninfected allantoic membrane		<1:4

The results of the neutralization test are quite clear cut, as shown in table 6. Unfortunately, acute serum was not available for comparative testing. Serum from another patient with a local NDV infection was tested. This serum, which was negative in the complement fixation and hemagglutination-inhibition tests, was also shown to be negative in the neutralization test.

TABLE 6.—*Neutralization of NDV by patient's serum 2 months after infection.*

Virus dilution	No. LD_{50}	Serum dilutions				
		Undiluted	1:23	1:50	1:100	1:500
10^{-4}	10^{3}	1*	1	1	1	1
10^{-5}	10^{4}	1	1	1	1	1
10^{-6}	10^{5}	2	2	1	1	1
10^{-10}	10^{9}	2	2	2	1	2

* Numerator is no. of embryos infected; denominator is no. of embryos harvested.

DISCUSSION

Following accidental introduction of the NDV into the human eye, thorough washing immediately afterward with water for 20 to 30 minutes and hourly washings for an additional 12 hours with 4% boric acid solution failed to prevent infection. In fact, it is possible that the prolonged washing may have enhanced the chances of infection.

Since other workers have isolated NDV from conjunctival washings on the first day that symptoms were noted, the virus was very probably present in the first washings from the case reported here but was retained by the Seitz filter pad. It is significant that virus was recovered from washings taken as late as the 6th day and that it could not be recovered on the 18th day.

The systemic manifestations of this disease would indicate that Newcastle disease is not always a localized infection limited to the conjunctiva. Successful isolation of the virus from the blood serum 7 hours after the chill and fever is evidence that NDV can produce a viremia.

Demonstration of particles in the cytoplasm of cells in the epithelial scrapings of the 2nd and 5th day after infection and the lack of such particles

in the epithelial cells of the 17th day suggest that NDV is capable of producing inclusion bodies. The transient nature of these microscopically discernible particles is characteristic of inclusion bodies formed in numerous other virus diseases. Since this is a new finding in Newcastle disease of man, and inclusion bodies have not been conclusively demonstrated in the natural host, definite proof that these particles are virus inclusion bodies rests upon subsequent demonstration of such particles in experimentally induced infection as well as the naturally occurring disease of fowls.

The immune response to NDV infection in the human has previously been reported[7,9,12] to be detectable but was usually of a relatively insignificant nature. In the case herein reported the immune response appears to follow the same pattern as has been demonstrated for other virus diseases such as mumps and influenza.

Serologic tests have been performed on two additional individuals from whom NDV was isolated, a poultry handler[13] on whom studies were done at Ohio State University and an animal attendant at The Virus Diagnostic Laboratory. The former had no complement-fixing antibodies when tested one year later. The latter's serums gave negative hemagglutination inhibition, neutralization, and complement fixation reactions when tested at 1, 4 and 11 weeks after onset of the conjunctivitis. The reason for the negative results in the first patient may be due to the delay in obtaining serums. The second patient had a very mild infection which may account for the completely negative serology.

SUMMARY

Newcastle disease virus was isolated from the eye washings and blood serum of an accidentally infected patient. Following a definite systemic reaction there was an immune response similar to those observed in certain other virus diseases. Inclusion bodies were noted in the cytoplasm of epithelial scrapings from the conjunctiva, but the significance of these findings has not yet been fully established.

EXPERIMENTAL HUMAN SALMONELLOSIS

I. PATHOGENICITY OF STRAINS OF *SALMONELLA MELEAGRIDIS* AND *SALMONELLA ANATUM* OBTAINED FROM SPRAY-DRIED WHOLE EGG

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Within recent years, the number of recognized *Salmonella* types has increased enormously. Many of these types have never been isolated from cases of human illness, while others have been found infrequently. Consequently, whether many of these types are pathogenic for man is not known. In species of proved pathogenicity, it is not certain that all strains or strains from all sources are pathogenic. Many species of *Salmonella* have been isolated from eggs and egg products. The pathogenicity of some of these species for the human is unquestionable, that of others is not fully known. A species may be recognized as a human pathogen, but the question still exists whether the strains recovered from eggs and egg products are pathogenic. The present investigation was undertaken to determine the pathogenicity for man of certain *Salmonella* strains isolated from spray-dried whole egg powder.

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Grateful acknowledgement is made to Dr. Mathilde Solowey of the Microbiology Research Division, Bureau of Agricultural and Industrial Chemistry, for her active interest and cooperative support throughout these studies. The strains of *Salmonella* used in these investigations were isolated and supplied by her laboratory. Dr. Gail M. Dack, Director of the Food Research Institute of the University of Chicago, has maintained an active and constructive interest in these studies. We are also grateful to Dr. P. R. Edwards, U. S. Public Health Service, for supplying generous amounts of the appropriate specific *Salmonella* typing serums.

MATERIALS AND METHODS

Selection of human volunteers.—Human volunteers were secured at a nearby penal institution. Subjects selected for experiment were healthy males. Each man was given a complete physical examination. Abnormalities which might be regarded as increasing the risk involved or rendering evaluation of symptoms difficult excluded an individual from the study. Medical histories of these men were available to us. In most instances the record extended over several years. Chronic complainers and those who had had frequent gastrointestinal disturbances in the past were eliminated. Food handlers were not accepted. After this initial selection, at least three weekly stool cultures were done. Only those individuals having no *Salmonella* or other easily confused organisms in the stools were carried further. An initial serum agglutination test was done against the organism to be administered. Subjects showing moderate or high agglutination titers against a particular organism were, in general, not used in the experiments with that species.

Source of Salmonella *strains.*—Strains of *Salmonella* used in this investigation were isolated from market samples of high moisture (4% to 6%) spray-dried whole egg powder by the Microbiology Research Division of the Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture.[1] After identification and one or more transfers, these cultures were lyophilized and stored until used in the present studies. Data obtained with three strains of *Sal-*

1. Solowey, M., McFarlane, V. H., Spaulding, E. H. and Chemerda, C. 1947, Am. J. Pub. Health. **37**: 971–982.

monella meleagridis and three strains of *Salmonella anatum* are reported in this paper. Subsequent communications will deal with other species under investigation.

Method of feeding.—Cultures selected for feeding were subcultured on trypticase soy agar (B-B-L). After 24 hours of incubation, the resulting growth was suspended in saline and standardized turbidimetrically (photoelectric colorimeter). The calculated dose was administered in a glass of eggnog shortly following the noon meal. The menstruum and the suspension of organisms were transported to the institution separately, and immediately prior to feeding the estimated amount of suspension for each individual dose was mixed with a glass of eggnog. At the same time, serial dilutions of the suspension of organisms were plated on trypticase soy agar in duplicate for final determination of the bacterial count. A group of men, usually consisting of 6, received the same experimental feeding.

Control feedings were provided by eggnog alone or by prior feedings of the test organisms at noninfective levels.

Observation after feeding.—Following each feeding the men were interviewed and observed three times a week for a period of two weeks and once a week or oftener thereafter. Additional visits were made when warranted by the condition of hospitalized patients. At each visit, the men were questioned in regard to symptoms, their temperatures were recorded, and fecal cultures were obtained. When indicated, blood counts and blood cultures were done. Blood samples for agglutination tests were drawn at weekly intervals for 4 weeks or longer.

Fecal cultures were obtained by inserting a gloved finger well within the rectum. Upon withdrawal, fecal material on the glove was removed with a sterile swab for culture. In most instances a gross fecal specimen was obtained. Cultures were done on all men three times a week for the first two weeks. Thereafter they were made once a week until at least three consecutive negative specimens had been obtained.

Hospitalization was provided if demanded by the degree of illness, otherwise no restrictions were placed upon the men. Subjects who did not become ill or develop transient carrier states were usually used in subsequent experiments as detailed in the tables.

Patients in whom illness developed were given no specific treatment and a minimum of symptomatic treatment. The natural course of illness was observed and studied. Supportive treatment was of course given in those few cases where it was medically indicated.

Cultural methods and identification.—Both direct and enrichment culture methods were employed. Direct cultures were made by streaking Shigella-Salmonella (SS) agar (Difco) and bismuth sulphite agar (Difco) plates with fecal material obtained at rectal examination. Two enrichment broths, Selenite F (B-B-L) and Tetrathionate Broth (Difco) containing brilliant green to 1:100,000, were also seeded. After 18 hours of incubation each enrichment broth was streaked out on 5 different plate mediums—SS agar (Difco), E. M. B. agar (Difco), bismuth sulphite agar (Difco), desoxycholate-citrate agar (Difco), and MacConkey agar (Difco). Culture plates were incubated at 37 C for 24 hours or longer and distinctive colonies selected for transfer to triple sugar iron (T. S. I.) agar (B-B-L). Cultures resembling *Salmonella* were tentatively identified by agglutination with a group specific serum. Subsequently, all isolates giving the characteristic biochemical reactions were specifically typed according to the Kauffmann-White schema (after Edwards and Bruner[2]).

Agglutination tests.—Agglutination tests on the subjects' serums were performed with standardized formalinized antigens made from the strains under study. In experiments with any strain, antigen made with that particular strain was employed. The same lot of antigen was used throughout the tests with each strain. All tests were read after incubation at 37 C for 24 hours. Serial dilutions of serum were employed, the lowest being 1:20.

RESULTS

Salmonella meleagridis

Results of feeding of the three strains of *S. meleagridis* are summarized in tables 1, 2, and 3. Subjects who became ill are starred in these tables and the cases are described in detail in the section on Description of Clinical Cases.

Because of the risk involved in this type of work, at the start of the investigation extremely small dosages were used initially, and conservative increments were used in subsequent feedings. In table 1 which presents the data derived with *S. meleagridis*, strain I, it will be noted that even with the initial

2. Edwards, P. R. and Bruner, D. W. 1942, Univ. Kentucky Agr. Exper. Station Circ. 54.

TABLE 1.—*Experimental feeding of* S. meleagridis, *strain I, to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer: initial	Aggl. titer: max.	Fecal cultures: No. of times isolated	Fecal cultures: Day last recovered
1	0	12,000	—†	—	0	0
2	0	12,000	1:20	1:20	2	4
3	0	12,000	—	—	1	4
4	0	12,000	—	—	1	2
5	0	12,000	—	—	0	0
6	0	12,000	—	—	0	0
7	0	24,000	—	—	3	9
8	0	24,000	1:40	1:40	2	4
9	0	24,000	1:20	1:20	0	0
10	0	24,000	—	—	0	0
11	0	24,000	—	—	2	4
12	0	24,000	1:40	1:40	1	2
13	0	52,000	—	—	0	0
14	0	52,000	—	—	0	0
15	0	52,000	1:20	1:20	1	4
16	0	52,000	—	—	7	21
17	0	52,000	—	—	2	4
18	0	52,000	1:20	1:20	0	0
19	2	96,000	1:20	1:20	1	2
20	2	96,000	—	—	0	0
21	2	96,000	—	—	1	2
22	2	96,000	—	—	2	7
23	2	96,000	—	—	0	0
24	0	96,000	—	—	0	0
25	3	155,000	1:20	1:20	0	0
26	3	155,000	—	—	2	4
27	3	155,000	—	—	1	2
28	3	155,000	—	—	1	21
29	3	155,000	—	—	5	12
30	1	155,000	—	—	8	40
31	2	300,000	—	—	1	2
32	2	300,000	1:40	1:40	1	2
33	2	300,000	1:20	1:20	12	42
34	2	300,000	—	—	1	4
35	2	300,000	1:40	1:40	4	11
36	0	300,000	—	—	1	2
37	2	720,000	—	—	2	4
38	2	720,000	—	—	0	0
39	2	720,000	1:20	1:20	1	2
40	2	720,000	—	—	5	11
41	2	720,000	1:20	1:40	6	16
42	0	1,145,000	1:20	1:20	4	9
43	0	1,145,000	—	—	2	4
44	0	1,145,000	1:40	1:40	5	11
45	0	1,145,000	—	—	5	11
46	0	1,145,000	—	—	11	25
47	0	1,145,000	1:320	1:320	1	2
48	0	5,500,000	—	—	2	4
49	0	5,500,000	—	—	11	42
50	0	5,500,000	—	—	0	0
51	0	5,500,000	1:40	1:40	12	42
52	0	5,500,000	1:20	1:20	2	4
53	0	5,500,000	1:20	1:20	1	2
54	3	24,000,000	1:40	1:40	7	25
*55	0	24,000,000	1:20	1:160	16	72
56	0	24,000,000	1:20	1:20	6	25
57	0	24,000,000	1:20	1:20	3	4
58	0	24,000,000	1:40	1:40	5	11
*59	1	50,000,000	1:20	1:40	6	14
*60	1	50,000,000	—	1:20	3	7
*61	1	50,000,000	1:40	1:40	4	11
*62	1	50,000,000	—	—	6	14
63	1	50,000,000	1:320	1:640	6	14
64	0	50,000,000	—	—	5	11

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.
† — = negative.

TABLE 2.—*Experimental feeding of* S. meleagridis, *strain II, to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer: initial	Aggl. titer: max.	Fecal cultures: No. of times isolated	Fecal cultures: Day last recovered
65	0	1,000,000	1:320	1:640	4	11
66	0	1,000,000	1:80	1:80	20	128
67	0	1,000,000	1:20	1:20	3	7
68	0	1,000,000	1:80	1:80	4	11
69	0	1,000,000	1:20	1:20	1	2
70	0	1,000,000	1:40	1:40	12	60
71	0	5,500,000	1:20	1:20	6	14
72	0	5,500,000	1:40	1:40	5	14
73	0	5,500,000	1:40	1:80	9	46
74	0	5,500,000	1:640	1:1280	2	4
75	0	5,500,000	1:40	1:40	1	2
76	0	5,500,000	1:160	1:160	2	4
*77	0	10,000,000	1:40	1:320	6	35
78	1	10,000,000	1:20	1:20	0	0
79	1	10,000,000	—	1:20	2	8
80	1	10,000,000	1:40	1:80	3	8
81	1	10,000,000	1:40	1:40	3	11
82	1	10,000,000	1:40	1:40	1	2
83	1	20,000,000	1:40	1:40	2	7
84	1	20,000,000	1:80	1:80	1	2
*85	1	20,000,000	—	—	6	32
*86	0	20,000,000	1:20	1:20	3	7
87	0	20,000,000	1:80	1:80	8	44
88	0	20,000,000	1:80	1:80	12	89
*89	1	41,000,000	1:320	1:640	5	10
*90	1	41,000,000	1:20	1:80	6	13
*91	1	41,000,000	1:80	1:80	6	13
*92	1	41,000,000	1:20	1:40	5	13
*93	0	41,000,000	1:40	1:160	6	20
94	0	41,000,000	1:40	1:80	5	34

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

TABLE 3.—*Experimental feeding of* S. meleagridis, *strain III, to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer: initial	Aggl. titer: max.	Fecal cultures: No. of times isolated	Fecal cultures: Day last recovered
95	1	158,000	1:40	1:40	0	0
96	1	158,000	1:40	1:40	0	0
97	1	158,000	1:640	1:640	0	0
98	0	158,000	1:20	1:20	1	2
99	0	158,000	1:80	1:80	0	0
100	0	158,000	1:40	1:40	0	0
101	0	1,500,000	1:40	1:40	0	0
102	0	1,500,000	1:20	1:40	3	9
103	0	1,500,000	1:40	1:40	3	7
104	0	1,500,000	1:40	1:40	2	4
105	0	1,500,000	1:20	1:40	5	21
106	0	1,500,000	1:20	1:20	1	2
107	1	7,675,000	1:40	1:40	2	7
108	1	7,675,000	1:80	1:80	3	7
109	0	7,675,000	1:40	1:40	8	30
110	0	7,675,000	1:40	1:40	3	9
111	0	7,675,000	1:160	1:160	9	37
*112	0	7,675,000	1:80	1:80	6	14
113	1	10,000,000	1:20	1:20	1	2
114	1	10,000,000	1:20	1:20	2	4
*115	1	10,000,000	1:20	1:20	2	4
*116	1	10,000,000	1:80	1:80	1	2
117	1	10,000,000	1:40	1:40	1	2
118	1	10,000,000	1:40	1:40	0	0

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

dosage of 12,000 organisms, fecal cultures were positive from three subjects. With increasing dosages, positive cultures were obtained from a greater percentage of the subjects, and transient carrier states persisted longer. No clinical illness resulted until the 10th feeding with a dosage of 24,000,000 organisms. In this group of 5 men, one became ill. When fed at a dosage of 50,000,000 organisms, 4 of the 6 men became ill. At every level fed, 50% or more of the men excreted organisms in the stool for variable periods. With all feedings of 155,000 or more, only occasional individuals failed to demonstrate organisms in the feces, to wit, three out of 40 subjects. A summary of the carrier states is presented in table 7.

The number of organisms required to produce illness with strain II was somewhat less than with strain I, one case occurring after receiving 10,000,000 organisms. Of the 30 men fed, only one individual failed to excrete organisms in the feces, and three individuals carried the organism for 60, 89, and 128 days, respectively (tables 2 and 7).

With strain III clinical illnesses were produced at an even lower level, one man becoming ill after receiving 7,675,000 organisms and two after receiving 10,000,000 organisms. At the initial feeding of 158,000 organisms only one man excreted *Salmonella* in the feces and this on the second day sampling only. In the three subsequent feedings, all except two of 18 individuals excreted organisms, with two men doing so for 30 and 37 days (tables 3 and 7).

In all subjects fed this species other than the 16 cases starred in tables 1, 2, and 3, no evidence of clinical illness was apparent at any time. None of the men had definitive symptoms attributable to the feedings, nor exhibited significant elevation of agglutination titer, even though carrier states persisted in some for many weeks.

In the cases of clinical illness described under that heading, 8 of the 16 showed no change in agglutination titer, 4 showed a rise of one dilution which was not considered significant, one showed a rise from 1:20 to 1:80, one from 1:40 to 1:160, one from 1:20 to 1:160, and one from 1:40 to 1:320.

Salmonella anatum

Table 4 presents the data obtained from the feeding of *S. anatum*, strain I, at 8 dosage levels. These feedings were conducted simultaneously with those of strain I, *S. meleagridis*, and similar small initial doses and small increments were employed. Here again, even with the initial dose of 12,000 organisms, positive fecal cultures were obtained. One of the men excreted the organism for 14 days. Tables 4 and 7 detail the carrier states produced with this strain. Five cases of illness resulted, two at the 587,000 dosage level and three at the 860,000 level.

Table 5 presents the data obtained from the feeding of *S. anatum*, strain II, at 8 dosage levels. Positive fecal cultures were again recovered consistently at all levels. In all but 6 of 50 individual feedings, organisms were recovered and in one man persisted for as long as 107 days. Illness was not produced until 44,500,000 organisms were fed, when one of 6 individuals became mildly ill. At the next level with 67,250,000 organisms, 4 out of 8 men became moderately ill. It will be noted that the infective dosage of this strain is markedly larger than that of strain I.

The results of feeding of *S. anatum*, strain III, are presented in table 6. Three dosage levels were used. The second level of 1,257,000 organisms produced illness in two men, one of whom

TABLE 4.—*Experimental feeding of* S. anatum, *strain I, to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer: initial	Aggl. titer: max.	Fecal cultures: No. of times isolated	Fecal cultures: Day last recovered
119	0	12,000	—	—	3	14
120	0	12,000	1:20	1:20	0	0
121	0	12,000	—	—	0	0
122	0	12,000	—	—	1	5
123	0	12,000	1:20	1:40	0	0
124	1	24,000	—	—	1	2
125	1	24,000	1:20	1:20	0	0
126	1	24,000	—	—	0	0
127	1	24,000	—	—	1	2
128	1	24,000	1:20	1:20	0	0
129	0	24,000	—	—	1	2
130	0	66,000	—	—	1	2
131	0	66,000	—	—	1	2
132	0	66,000	—	—	6	14
133	0	66,000	1:40	1:40	0	0
134	0	66,000	—	—	0	0
135	0	66,000	1:20	1:40	5	12
136	2	93,000	—	—	0	0
137	2	93,000	1:20	1:20	6	15
138	0	93,000	1:20	1:20	0	0
139	2	93,000	1:20	1:40	0	0
140	1	93,000	—	—	0	0
141	0	93,000	—	—	0	0
142	1	141,000	—	—	0	0
143	1	141,000	—	—	1	2
144	1	141,000	1:40	1:40	2	4
145	1	141,000	—	—	0	0
146	1	141,000	1:20	1:40	3	7
147	0	141,000	1:40	1:40	0	0
148	3	256,000	—	—	4	11
149	3	256,000	1:20	1:20	2	7
150	1	256,000	1:20	1:40	1	4
151	3	256,000	1:20	1:40	0	0
152	2	256,000	—	—	1	2
153	1	256,000	—	—	7	16
*154	0	587,000	1:40	1:80	9	37
155	0	587,000	—	—	2	5
156	0	587,000	1:20	1:20	0	0
157	0	587,000	1:20	1:40	1	2
*158	0	587,000	1:20	1:40	1	2
159	0	587,000	—	—	0	0
160	2	860,000	1:20	1:40	1	14
161	3	860,000	—	1:20	5	11
*162	0	860,000	1:40	1:640	7	25
*163	0	860,000	1:20	1:80	6	14
*164	0	860,000	1:20	1:40	6	14
165	0	860,000	—	1:20	2	4

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

TABLE 5.—*Experimental feeding of* S. anatum, *strain II, to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer: initial	Aggl. titer: max.	Fecal cultures: No. of times isolated	Fecal cultures: Day last recovered
166	2	89,000	—	—	3	14
167	2	89,000	1:40	1:40	1	11
168	0	89,000	—	—	2	11
169	0	89,000	1:40	1:40	1	7
170	0	89,000	—	—	0	0
171	0	89,000	1:40	1:40	4	9
172	0	448,000	1:40	1:40	0	0
173	0	448,000	1:40	1:80	2	4
174	0	448,000	1:40	1:40	1	2
175	0	448,000	1:80	1:80	1	2
176	0	448,000	1:40	1:40	1	2
177	0	448,000	1:20	1:20	0	0
178	1	1,042,500	1:40	1:40	3	14
179	1	1,042,500	—	1:20	5	11
180	1	1,042,500	—	—	1	2
181	1	1,042,500	1:40	1:40	1	2
182	1	1,042,500	1:20	1:40	1	2
183	1	1,042,500	1:20	1:20	2	11
184	1	3,900,000	—	—	5	21
185	1	3,900,000	1:40	1:40	0	0
186	1	3,900,000	1:20	1:20	1	2
187	1	3,900,000	1:40	1:40	9	32
188	0	3,900,000	1:40	1:40	0	0
189	0	3,900,000	—	1:20	7	21
190	1	10,000,000	1:40	1:80	2	8
191	1	10,000,000	1:40	1:80	3	8
192	1	10,000,000	1:40	1:40	1	2
193	1	10,000,000	1:80	1:80	2	8
194	1	10,000,000	1:40	1:40	1	2
195	1	10,000,000	1:40	1:40	1	8
196	2	23,900,000	1:40	1:40	3	7
197	2	23,900,000	—	—	0	0
198	2	23,900,000	—	—	1	2
199	0	23,900,000	1:40	1:80	2	7
200	0	23,900,000	1:40	1:40	13	107
201	0	23,900,000	1:40	1:40	1	2
202	2	44,500,000	1:40	1:80	3	6
203	2	44,500,000	1:40	1:80	1	3
204	2	44,500,000	1:40	1:40	3	6
205	0	44,500,000	1:80	1:80	3	6
*206	0	44,500,000	1:40	1:40	3	6
207	0	44,500,000	1:80	1:160	3	10
208	2	67,250,000	1:40	1:40	4	11
209	2	67,250,000	1:20	1:20	1	2
210	2	67,250,000	1:80	1:80	2	4
*211	2	67,250,000	1:40	1:40	6	14
*212	1	67,250,000	1:80	1:80	2	4
*213	1	67,250,000	1:20	1:20	3	5
*214	1	67,250,000	1:40	1:80	4	9
215	1	67,250,000	1:20	1:20	3	11

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

required hospitalization. At the third level of 4,675,000 organisms, 4 of the 6 men fed became ill, two seriously enough to require hospitalization.

Sixteen individuals fed *S. anatum* became ill. All other subjects failed to show any evidence of clinical illness at any time. Even those carrying the organism for several weeks had no symptoms, and there was no significant change in their agglutination titers. Of those becoming ill, 6 showed no change in the agglutination titer, 5 an insignificant rise of one dilution, two increased from 1:20 to 1:80, two from 1:40 to 1:160, and one from 1:40 to 1:640.

DESCRIPTION OF CLINICAL CASES

The 32 cases of clinical illness observed during these feeding experiments vary greatly in respect to incubation period, severity, duration, and clinical characteristics. Twelve patients were ill enough to warrant hospitalization. The others remained ambulatory in most instances, a few

TABLE 6.—*Experimental feeding of* S. anatum, *strain III, to human volunteers.*

Feeding no.	No. of previous feedings	No. of organisms fed	Aggl. titer: initial	Aggl. titer: max.	Fecal cultures: No. of times isolated	Fecal cultures: Day last recovered
216	2	159,000	—	—	0	0
217	2	159,000	1:40	1:40	0	0
218	2	159,000	1:20	1:40	2	9
219	0	159,000	1:80	1:80	0	0
220	0	159,000	1:20	1:40	3	9
221	0	159,000	1:40	1:40	0	0
222	0	1,257,000	1:20	1:40	2	9
223	0	1,257,000	1:160	1:160	4	9
*224	0	1,257,000	1:20	1:80	5	11
225	0	1,257,000	1:80	1:80	7	28
*226	0	1,257,000	1:40	1:40	5	14
227	0	1,257,000	1:160	1:160	2	21
*228	2	4,675,000	1:40	1:160	8	21
229	1	4,675,000	1:40	1:40	3	21
*230	1	4,675,000	1:40	1:40	8	30
*231	0	4,675,000	1:80	1:160	3	7
232	0	4,675,000	1:80	1:80	2	14
*233	0	4,675,000	1:40	1:160	9	30

* Clinical illness described under section on DESCRIPTION OF CLINICAL CASES.

being given lay-in privileges in their cells. In some instances ambulatory observation was carried out because of the subject's strong aversion and resistance to hospitalization.

In some of the cases described here, the degree of illness is quite minor and not too well supported by objective evidence. Verification for these cases is amply provided by objectively substantiated illness of those fed the same dose, or in some instances the next larger dose. Further, control feedings or prior feeding of nonpathogenic dose levels did not elicit symptoms in these individuals. The inclusion of these cases is desirable to clearly delineate the wide range of clinical illness which may be produced by these organisms.

Salmonella meleagridis.—No. 55 received 24,000,000 organisms of *S. meleagridis*, strain I. Twenty to 22 hours later, he developed generalized aching and chilliness and was hospitalized. The temperature was normal. Twenty-six hours after the feeding, diarrhea commenced. There were 22 loose watery stools during the next 24 hours. Abdominal cramps and nausea accompanied the diarrhea. He vomited 5 times. During the first day of hospitalization, the maximum temperature recorded was 100.4 F. Temperatures thereafter remained normal. The white blood count on the day of admission was 11,100 with 86% polymorphonuclears. The succeeding day it was 8400 with 75% polymorphonuclears. During the second day of illness there were 4 loose watery stools. Thereafter, the patient continued to improve but complained of intermittent abdominal distress, cramps, flatulence, and occasional watery stools for the next two weeks. Two blood cultures remained sterile. The agglutination titer rose from 1:20 at the start of the experiment to 1:160. The organism was last recovered from the stool 72 days after feeding.

No. 59 received 50,000,000 organisms of *S. meleagridis*, strain I. Twenty to 22 hours later he developed abdominal cramps and diarrhea. During the ensuing 24 hours there were 8 loose watery stools. There was no nausea, vomiting, or chills. He was admitted to the hospital 48 hours after feeding at which time the temperature was 101.8 F, and the white blood count was 10,900 with 72% polymorphonuclears. The highest recorded temperature during the second day of illness was 101.6 F, and during the third 99.6 F. After the first day there was no further diarrhea. During the second day of illness there were no bowel movements, and on the third there were two normal stools. Thereafter, bowel function and temperature remained normal and the patient promptly recovered. A blood culture remained sterile. The agglutination titer changed from 1:20 to 1:40. The organism was last recovered from the stool 14 days after feeding.

No. 60 received 50,000,000 organisms of *S. meleagridis*, strain I. Twenty-four hours later he became ill rather suddenly starting with a chill

TABLE 7.—*The recovery of* Salmonella *from fecal cultures after experimental feeding.*

Organism	No. of feedings	No. of men with positive cultures	% with positive cultures	Duration of positive cultures: <1 week	1 to 2 weeks	2 to 3 weeks	3 to 4 weeks	>4 weeks	% >4 weeks	Av. duration (days)	Longest duration (days)
S. meleagridis strain I	64	50	78.1	24	12	4	5	5	10.0	12.1	72
S. meleagridis strain II	30	29	96.7	6	12	3	0	8	27.6	22.4	128
S. meleagridis strain III	24	17	70.8	8	5	1	1	2	11.8	9.6	37
Total	118	96	81.4	38	29	8	6	15	15.6	14.7	
S. anatum strain I	47	28	59.4	14	5	7	1	1	3.6	9.0	37
S. anatum strain II	50	44	88.0	21	16	3	2	2	4.5	9.9	107
S. anatum strain III	18	14	77.7	0	6	2	3	3	21.4	16.7	30
Total	115	86	74.8	36	27	12	6	6	7.0	10.7	

followed by headache, nausea, vomiting, weakness, abdominal cramps, and diarrhea. Upon admission to the hospital two and one-half hours after the chill, the temperature was 101.6 F. There were three watery stools during the first six hours of hospitalization, and no further diarrhea thereafter. The white blood count was 6700 with 50% polymorphonuclears. During the second day of illness the highest recorded temperature was 99.2 F, and recordings thereafter were normal. The patient continued to complain of extreme weakness and lack of appetite for several more days. He gradually improved for the next 10 days to complete recovery. A blood culture remained sterile. The agglutination titer changed from negative to 1:20. The organism was last recovered from the stool 7 days after feeding.

No. 61 received 50,000,000 organisms of *S. meleagridis*, strain I. Eighteen hours later he complained of headache and generalized aching. Twenty-eight hours after feeding he developed a chill followed by nausea and diarrhea, with four watery stools in the next 12 hours. The temperature was 99.1 F. There was no further diarrhea, but he had several soft formed stools per day for the next three days, and thereafter bowel function was normal. He continued slightly febrile with elevations of temperature of 99.2 F to 99.6 F for 5 days. The white blood count on the first day of illness was 5800 and on the third day was 4100. He was completely recovered 6 days after the onset. A blood culture remained sterile. There was no rise in the agglutination titer. The organism was last recovered from the stool 11 days after feeding.

No. 62 received 50,000,000 organisms of *S. meleagridis*, strain I. About 36 to 40 hours later he experienced mild abdominal cramps and chilliness and had one loose watery stool. Forty-eight hours after feeding the temperature was 99.8 F, and the white blood count was 6100 with 57% polymorphonuclears. There were two watery stools during the next 24 hours. The temperature was slightly elevated for two days. Thereafter there were no further complaints. There was no rise in the agglutination titer. The organism was last recovered from the stool 14 days after feeding.

No. 77 received 10,000,000 organisms of *S. meleagridis*, strain II. About 10 to 12 hours later diarrhea commenced. During the next 36 hours there were approximately 16 loose watery stools. The onset of diarrhea was accompanied by severe abdominal cramping, but this subsided by the next morning. As he did not feel ill, although he continued to have loose stools, he did not report to the hospital. The temperature was first obtained 48 hours after feeding, at which time it was 99.2 F. Diarrhea had ceased and he felt better. The agglutination titer subsequently rose from 1:40 to 1:320. The organism was last recovered from the stool 35 days after feeding.

No. 85 received 20,000,000 organisms of *S. meleagridis*, strain II. Twenty hours later he developed abdominal cramps and diarrhea with 7 watery stools during the day. The temperature remained normal throughout. The white blood count was 6500. There was no nausea, vomiting, or chills. The following morning he felt well and had no further complaints. There was no change in the agglutination titer. The organism was last recovered from the stool 32 days after feeding.

No. 86 received 20,000,000 organisms of *S. meleagridis*, strain II. Twenty-two hours later he developed abdominal cramps, chilliness, and diarrhea with 4 watery stools during the day. The temperature was normal and remained so. The white blood count was 5100. The following morning and thereafter he felt well. The agglutination titer was unchanged. The organism was last recovered from the stool 7 days after feeding.

No. 89 received 41,000,000 organisms of *S. meleagridis*, strain II. About 26 hours later he developed slight nausea and abdominal cramps. The temperature was normal. Five to 6 hours later he felt chilly and feverish and vomited two or three times. Diarrhea commenced and persisted for two days with approximately 40 watery stools during this time. Although the patient felt feverish at the onset of diarrhea, the temperature was not obtained at this time. The next morning upon admission to the hospital it was normal and remained so thereafter. Four days after the onset of illness he had completely recovered and gave no further complaints. The agglutination titer changed from 1:320 to 1:640. The organism was last recovered from the stool 10 days after feeding.

No. 90 received 41,000,000 organisms of *S. meleagridis*, strain II. Thirty hours later he developed mild abdominal cramps and diarrhea. During the next 6 hours he felt chilly and feverish. He vomited three times and had 8 to 10 watery stools. The second day there were 8 watery stools, the succeeding day three. Abdominal cramps persisted. Recorded temperatures remained in the normal range. Subsequent to 4 days after feeding there were no further complaints. The agglutination titer rose from 1:20 to 1:80. The organism was last recovered from the stool 13 days after feeding.

No. 91 received 41,000,000 organisms of *S. meleagridis*, strain II. Thirty hours later he developed abdominal cramps, felt chilly, and

vomited three times. Diarrhea developed with 12 watery stools during the first 24 hours and continued at a lesser degree for an additional 48 hours. Abdominal cramps continued for three days. Thereafter there were no complaints. There were no abnormalities in the recorded temperatures. There was no change in the agglutination titer. The organism was last recovered from the stool 13 days after feeding.

No. 92 received 41,000,000 organisms of *S. meleagridis*, strain II. The third and fourth day following he had three or four watery stools daily. No cramps, nausea, vomiting, or chilliness occurred, and the recorded temperatures were all normal. He was asymptomatic thereafter. There was a change in the agglutination titer from 1:20 to 1:40. The organism was last recovered from the stool 13 days after feeding.

No. 93 received 41,000,000 organisms of *S. meleagridis*, strain II. Seventy-two hours later he complained of mild abdominal cramping. The temperature at this time was normal. He continued to complain of abdominal cramping for several days, although bowel movements were normal. On the 6th day after feeding there were three loose watery stools and the temperature was 99 F. There were no further complaints. The agglutination titer subsequently rose from 1:40 to 1:160. At the time of observation, although this man's complaints were quite mild, he was classed as a questionable illness since 4 other men in the group of 6 were ill. The rise in agglutination titer would appear to validate this case. The organism was last recovered from the stool 20 days after feeding.

No. 112 received 7,675,000 organisms of *S. meleagridis*, strain III. Approximately 22 hours later he was admitted to the hospital with abdominal cramps, nausea, and diarrhea. The temperature was 100.8 F, rising later in the day to 101.4 F. The white blood count was 7400. The following day the patient was afebrile and asymptomatic and remained so thereafter. There was no change in the agglutination titer, and a blood culture remained sterile. The organism was last recovered from the stool 14 days after feeding.

No. 115 received 10,000,000 organisms of *S. meleagridis*, strain III. Fifty-two hours later he developed a chill, nausea, and abdominal cramps. During the ensuing 24 hours there were approximately 36 watery stools. Nausea persisted for three days and abdominal cramping with 2 to 4 loose stools daily, for 5 days. The highest temperature elevation recorded was 99 F, but the initial reading was not obtained until he was hospitalized, 24 hours after the chill. The patient slowly recovered over a period of one week. The agglutination titer remained unchanged. The organism was last recovered from the stool 4 days after feeding.

No. 116 received 10,000,000 organisms of *S. meleagridis*, strain III. Twenty-four hours later he developed nausea and severe abdominal cramping. In the next few hours there were 6 watery stools. The following morning he complained of abdominal rumbling but otherwise felt comfortable. On the third day he again complained of cramps and had 6 watery stools. Thereafter he remained well. There was no recorded temperature elevation. There was no change in the agglutination titer. The organism was recovered from the stool on the second day following feeding but not thereafter.

Salmonella anatum.—No. 154 received 587,000 organisms of *S. anatum*, strain I. Eighteen hours later he became ill with onset of headache, nausea, vomiting, and diarrhea. During the first day of illness he had 4 loose watery stools accompanied by intermittent abdominal cramps, and he vomited 6 times. The abdomen was tender throughout to palpation. The temperature was not obtained at the onset, but the next morning it was 99.6 F and the white blood count was 11,500. The second day of illness he complained of abdominal cramps and chilliness and there were three watery stools. He promptly recovered and was without symptoms, although regularly excreting the organism, until 30 days after feeding when he again became ill. At the onset of this recurrence he vomited once. During the next day there were 8 to 10 loose watery stools. The highest temperature during the first day of recurrent illness was 99.6 F, and the white blood count was 8100. Although ill, the patient resisted hospitalization in lieu of which he was given a three day lay-in in his cell. Diarrhea persisted with 3 to 4 watery stools daily and there was a low-grade fever. He was transferred to the hospital on the third day of illness. Convalescence progressed slowly, and he became afebrile 5 days after the onset. He was followed for 4 months after recovery and remained entirely well. Two blood cultures were sterile. The agglutination titer changed from 1:40 to 1:80. The organism was last recovered from the stool 37 days after feeding and 7 days after the onset of the recurrence of illness.

No. 158 received 587,000 organisms of *S. anatum*, strain I. Thirty-six hours later he felt chilly and nauseated but did not vomit. The temperature was 99 F. Temperatures taken thereafter were all normal. There were two loose watery stools per day for the succeeding two days. He complained of occasional abdominal cramps. Recovery was prompt. The agglutination titer changed from 1:20 to 1:40. The or-

ganism was recovered from the stool on but one occasion two days after feeding.

No. 162 received 860,000 organisms of *S. anatum*, strain I. Twenty-five hours later he became suddenly nauseated and vomited three times. Immediately following this, he had three watery stools. He was admitted to the hospital late in the afternoon. The temperature on admission was 98 F; the white blood count was 10,700. During the evening he vomited three more times, and there were 7 or 8 watery stools during the night. The temperature at midnight was 100.6 F. The temperature was taken every 4 hours and there were no normal readings for 4 days. The highest temperature during the first day of illness was 100.8 F, the second day 103 F, the third day 102.2 F, and the fourth day 101.8 F. All readings after the 4th day were normal. This patient appeared severely ill and prostrated. He complained of headache, weakness, nausea, abdominal cramps, and backache. He vomited occasionally during the first two days and had repeated chills. After the first 24 hours there was no further diarrhea. The white blood count on the third day of illness was 9600 with 73% polymorphonuclears. The patient received intravenous fluids and glucose daily for 4 days. He was unable to take anything by mouth. Occasional small doses of codeine were given for headache and generalized discomfort. The abdomen was tender to palpation. The spleen could not be felt.

After subsidence of nausea, diarrhea and fever, there was slow convalescence. He continued to complain of extreme weakness and abdominal soreness and discomfort with occasional severe abdominal cramps for another week. During this period there were two to four soft mushy stools per day but no frank diarrhea. Convalescence was judged to be complete three weeks after the onset of illness. Daily blood cultures taken during the febrile period remained sterile. The agglutination titer rose from 1:40 to 1:640. The organism was last recovered from the stool 25 days after feeding.

No. 163 received 860,000 organisms of *S. anatum*, strain I. Twenty-four hours later he developed diarrhea. There were 7 loose watery stools during the next 12 hours. He complained of chilliness, a flushed feeling, severe headache, and slight nausea. Upon admission to the hospital 36 hours after feeding, the temperature was 99.4 F, and the white blood count was 3700 with 41% polymorphonuclears. He continued to complain of headache, nausea, and occasional abdominal cramps. There was an average of three watery stools per day for the next three days. He remained slightly febrile for 5 days; the highest temperature recorded was 100.2 F. He vomited on several occasions. The abdomen was soft and not tender. The white blood count on the second day was 4000; on the third day 3500; and on the 6th day 5800. He was completely recovered 10 days from the onset of illness. Three blood cultures remained sterile. The agglutination titer rose from 1:20 to 1:80. The organism was last recovered from the stool 14 days after feeding.

No. 164 received 860,000 organisms of *S. anatum*, strain I. Forty-eight hours later he complained of malaise and generalized aching. Bowel movements were normal. The temperature was 100.2 F. Six hours later diarrhea commenced. There were 4 watery stools in the next 6 hours and the patient complained of nausea, chilliness, and profuse sweating. In the next three days there were 3 to 4 loose watery stools per day accompanied by abdominal aching and occasional cramps. There was low grade fever for two days, the temperature thereafter remaining normal. Six days after the onset of illness he was completely recovered. A blood culture remained sterile. The agglutination titer changed from 1:20 to 1:40. The organism was last recovered from the stool 14 days after feeding.

No. 206 received 44,500,000 organisms of *S. anatum*, strain II. Forty-eight hours later he developed abdominal cramps and diarrhea with 9 loose watery stools during the ensuing 24 hours. Recorded temperatures remained normal. There was then one day in which he had 4 loose watery stools with a return to normal function. There was no change in the agglutination titer. The organism was last recovered from the stool 6 days after feeding.

No. 211 received 67,250,000 organisms of *S. anatum*, strain II. Thirty-six hours later he developed mild abdominal cramps and diarrhea with 5 loose watery stools during the next 12 hours. There were no chills, nausea, vomiting, or headache. The temperature 12 hours after the onset of illness was 100.2 F, and the white blood count was 4800. There was no further diarrhea, and two days later the patient felt well. After the first day there was no temperature elevation. There was no change in the agglutination titer. The organism was last recovered from the stool 14 days after feeding.

No. 212 received 67,250,000 organisms of *S. anatum*, strain II. Sixteen to 20 hours later he developed nausea and abdominal distress. Bowel movements remained normal. The next day he complained of slight headache and abdominal soreness. The temperature was 99.4 F, and the white blood count was 4400. He was given a two day lay-in in his cell. There were three soft

stools on the first day but no frank diarrhea. Mild abdominal cramps and soreness persisted for two days. After that he felt well. There was no change in the agglutination titer. The organism was last recovered from the stool 4 days after feeding.

No. 213 received 67,250,000 organisms of *S. anatum*, strain II. Approximately 30 hours later he developed abdominal cramps and loss of appetite. There were three watery stools during the next 24 hours. The white blood count was 7700. Recorded temperatures were all normal. There were no further complaints. The agglutination titer remained unchanged. The organism was last recovered from the stool 5 days after feeding.

No. 214 received 67,250,000 organisms of *S. anatum*, strain II. Thirty to 32 hours later he commenced to feel ill with chilliness and a flushed feeling. He slept poorly. His abdomen was sore. Approximately 40 hours after feeding he had two loose watery stools and developed headache. The temperature the next day was 99.6 F, and the white blood count was 6800. He refused food throughout the day. There was no further diarrhea, but intermittent abdominal cramping persisted for another 24 hours. The agglutination titer changed from 1:40 to 1:80. The organism was last recovered from the stool 9 days after feeding.

No. 224 received 1,257,000 organisms of *S. anatum*, strain III. Thirty hours later he developed generalized malaise, abdominal cramps and diarrhea. During the next 12 hours there were 5 loose watery stools. The temperature was not obtained until he was hospitalized 48 hours following feeding at which time it was 102 F. The white blood count was 7800. After the first hospital day the temperature remained normal. Diarrhea persisted for two days with 4 or 5 loose stools each day. Abdominal cramping likewise persisted for this time and malaise for several days more. Thereafter he was asymptomatic. Blood cultures remained sterile. The agglutination titer rose from 1:20 to 1:80. The organism was last recovered from the stool 11 days after feeding.

No. 226 received 1,257,000 organisms of *S. anatum*, strain III. Forty-eight hours later he was asymptomatic. His temperature at that time was 99.4 F, and the white blood count was 11,100. About 60 hours following feeding he developed abdominal cramps, and in the next 12 hours there were 5 loose watery stools. The temperature remained normal thereafter. Diarrhea ceased, and he recovered promptly. There was no change in the agglutination titer. The organism was last recovered from the stool 14 days after feeding.

No. 228 received 4,675,000 organisms of *S. anatum*, strain III. Nine hours later there was sudden onset of illness with chills followed by nausea and 6 watery stools during the night. He was admitted to the hospital 20 hours after feeding. At this time the temperature was 100 F, and during the afternoon it reached 102.6 F. The white blood count was 6300. During the first 24 hours in the hospital, he complained of abdominal cramps and had 12 loose watery stools. The following day his morning temperature was normal, the afternoon temperature was 99.6 F. He continued to complain of abdominal soreness and slight nausea. There were 6 watery stools on the second day after which diarrhea ceased. The third day the patient was afebrile, although he continued to complain of abdominal soreness for two more days. Blood cultures remained sterile. The agglutination titer rose from 1:40 to 1:160. The organism was last recovered from the stool 21 days after feeding.

No. 230 received 4,675,000 organisms of *S. anatum*, strain III. Thirty hours later he developed abdominal cramps, and in the next 12 hours there were three loose watery stools. The temperature reached 99 F for two days. There were no further complaints. The agglutination titer remained unchanged. The organism was last recovered from the stool 30 days after feeding.

No. 231 received 4,675,000 organisms of *S. anatum*, strain III. Between 30 and 36 hours later he felt chilly and had three loose stools during the night. The next morning the temperature was 99.4 F. The patient complained of abdominal cramps. The white blood count was 7500. There were no further complaints, diarrhea, or temperature elevations. The agglutination titer changed from 1:80 to 1:160. The organism was last recovered from the stool 7 days after feeding.

No. 233 received 4,675,000 organisms of *S. anatum*, strain III. Eight hours later he developed a chill, nausea, abdominal cramps, and diarrhea. There were 5 to 7 watery stools during the night. During the next day he continued to have nausea and abdominal cramps and 8 or 9 watery stools and was hospitalized at 8:00 P.M. The temperature was 101.8 F, and the white blood count was 10,000. Diarrhea and abdominal cramps persisted for 4 more days with 4 to 7 watery stools per day. Fever persisted for three days with peaks of 101.8 F, 101.4 F, and 99 F. Blood cultures remained sterile. The agglutination titer rose from 1:40 to 1:160. The organism was last recovered from the stool 30 days after feeding.

DISCUSSION

The advantages of using human volunteers living in a closed environment is obvious. The institution used in these studies has over 4000 inmates. The health record and sanitation are excellent. Since the prison population consists of repeaters or long-term criminals, little trouble has been experienced in following individuals for prolonged periods. The inmates mess in common. Digestive disturbances arising from causes outside the experiment could be readily evaluated. Individual or multiple cases of diarrhea or other digestive disturbances occurring in the institution were called to our attention, and we were accorded the privilege of studying them. Although several dozen such subjects were studied throughout the course of this work, we did not find a single instance of spontaneous salmonellosis due to the types we were using or to other types.

Opportunity was afforded to gain information about possible transfer from man to man. After experimental feeding, the subjects were not restricted from their usual activities. Hospitalization was provided only when warranted by clinical illness. Further, many subjects excreted organisms for prolonged periods. Cell mates of long-term carriers and of cases were examined in numerous instances. None of these individuals developed objective or subjective evidence of illness, and those tested showed no *Salmonella* in the stool and no rise in agglutination titer. Food handlers were of course excluded from the project. It must be remembered that whenever cases of intestinal disturbance occurred in this institution, we were allowed to study them. Certainly if any significant illness had resulted due to transfer from experimental subjects, it would have come to our attention. It is significant that no such case has been observed. It would appear then that the danger of transfer from person to person under the conditions of these experiments is small.

The primary aim of this work has been to determine whether the strains fed were capable of producing human illness. It was necessary then to adopt rather strict criteria of illness. Abdominal complaints are very common in presumably healthy people. Throughout the experiments it was necessary to differentiate between nonspecific complaints and those which might be specific. Subjective complaints were given little weight unless accompanied by objective evidence. For substantiation of specific illness in a subject fed *Salmonella*, in addition to subjective complaints and recovery of the organism from the stool, one should require one or more of the following: diarrhea or vomiting (preferably observed in the hospital), fever, rise in specific agglutination titer, or other observable signs. Many of our cases more than satisfied these minimal requirements.

Prior to administration of *Salmonella*, control feedings consisting of the menstruum alone were employed regularly in the earlier part of the work. Later, as the inmates learned via the "prison grapevine" that few of those fed became ill, such feedings were used less regularly. Men not developing either illness or transient carrier states on small dosages were fed again at higher levels. These previous feedings at noninfective levels serve as even better controls than the administration of menstruum alone. Over half of the subjects developing illness had received prior feedings at noninfective levels, and each infective dosage level included some of these individuals.

The incubation period in three-fourths of the cases was from 20 to 48 hours, in one it was as short as 8 hours,

and in another as long as 72 hours. The average of all cases of illness was 29.4 hours with no significant difference between the strains or the species.

A wide range of clinical illness was observed varying from mild diarrhea of one or two days' duration to a severe prostrating gastroenteritis with chills and high fever requiring two or three weeks for recovery.

Although all strains tested proved pathogenic, there was great variation in infectivity. Illness resulted with *S. meleagridis*, strain III, at 7.67 million and 10 million levels, whereas with strain I, 24 million and 50 million levels were required. With *S. anatum*, the range was even greater, strain I producing illness at 587 thousand and 860 thousand levels, while strain II required 44.5 million and 67.2 million levels.

The high incidence of excretion of organisms in the feces in the absence of symptoms emphasizes the question of the public health significance of the transient carrier. Although no cases of cross-infection were noted in our experiments, it must be remembered that food handlers were excluded from the project. Carrier states resulted from surprisingly small doses and in some individuals persisted for months.

From the results presented in tables 1 through 6 and the detailed description of the clinical illnesses, there can be no question that these three strains each of *S. anatum* and *S. meleagridis* derived from powdered egg are pathogenic for man. A bacterial count sufficient to produce clinical illness could conceivably result from reconstituted egg, particularly that prepared from high moisture powder (4% and above), which has been allowed to stand for several hours at room temperature, and not subsequently subjected to adequate heat treatment.[3,4]

SUMMARY

Three strains of *Salmonella meleagridis* and three strains of *Salmonella anatum* originally isolated from market samples of spray-dried whole egg powder were fed to human volunteers. All 6 strains produced human illness. Multiple dose levels were employed, and the minimal infective dose for man was determined for each strain. Clinical salmonellosis was produced in 32 men. The incubation period in three-fourths of the cases was from 20 to 48 hours and ranged from 8 to 72 hours. The cases varied in severity from mild brief enteritis to serious prostrating illness. Wide variation in the infective dose occurred from strain to strain of the same species. The number of organisms required to produce illness was in all instances within the range which might conceivably be encountered. Many cases of asymptomatic carrier states were produced, some persisting for many weeks.

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EXPERIMENTAL ARTHRITIS IN ALBINO RATS PRODUCED BY A STRAIN OF *CORYNEBACTERIUM*

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In a search for a suitable test organism to be used in studying the effects of antibiotics and chemotherapeutic agents on experimental infections of laboratory animals, a white mouse was found with a subcutaneous abscess on the back. The lesion appeared to be the result of a bite wound. The pus from this abscess was streaked on a blood agar plate and incubated at 37 C. Stained smears of the pus showed many Gram positive bacilli with diphtheroid morphology. The plate yielded a pure culture of a strain of *Corynebacterium*.

Description of the organism

The organism is a Gram positive, nonmotile bacillus three-tenths to six-tenths of a micron in diameter and one to three microns in length. The cells are straight or slightly curved and often club-shaped. Alternate bands of stained and unstained material appear when the cells are treated with Gram or methylene blue stains. Metachromatic granules may be demonstrated by Neisser's stain. Angular and palisade arrangement of the cells is frequent. On blood agar plates the organism produces round, convex colonies about one mm in diameter after 48 hours of incubation at 37 C. There is a slight greening of the medium. The colonies are white, opaque, and glistening with an even margin and smooth surface. Growth on unenriched nutrient agar is less abundant. In nutrient broth a granular turbidity and heavy sediment appear. No pellicle is formed. Gelatine is not liquefied. There is no visible growth on potato. On Loeffler slants there is moderate colorless growth, no peptonization. There is no change in litmus milk, and indol is not formed from tryptophane. Nitrite is produced from nitrate. The organism is aerobic. It grows anaerobically in the presence, but not in the absence of carbon dioxide. Esculin and sodium hippurate are hydrolyzed, but arginine is not. Hydrogen sulfide is not produced. The bacillus elaborates catalase. Acid but no gas is produced in broth containing glucose, levulose, maltose, mannose, sucrose, and starch. Salicin may or may not be fermented. No acid is produced from adonitol, amygdaline, arabinose, cellobiose, dextrine, dulcitol, erythritol, galactose, glycerol, inositol, inulin, lactose, mannitol, raffinose, rhamnose, trehalose, and xylose. Growth at 37 C is more luxuriant than at room temperature. Ammonia is not produced by growth in peptone water. No evidence of toxin production has been obtained. The organism was designated MT-9.

Three similar strains have since been isolated from throat cultures of normal stock mice and three strains have also been isolated from caseous lung abscesses of uninoculated white rats.

The experimental infection in mice

The organism is virulent for white mice when injected intravenously or intraperitoneally. Subcutaneous injection generally results in a localized abscess which drains and heals. The intravenous LD_{50} for 20 g white mice is approximately 10,000 viable bacilli as determined by plate count. Since the organism has a tendency to grow in packets which cannot be completely broken up by shaking, the plate count represents viable cells plus viable clumps. Mice given 200,000 viable bacilli intravenously die in from 3 to 8 days following injection. At autopsy the kidneys show multiple abscesses in which the diphtheroid bacilli may be demonstrated in large numbers by smear and culture. The liver frequently shows one or more localized abscesses. The lungs occasionally show abscesses large enough to be seen without magnification. Gross ab-

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scesses in other organs occur infrequently but have been observed in heart muscle, brain, and joints. All mice used were of the National Institutes of Health general purpose strain.

The experimental infection in rats

An attempt was made to determine the virulence of strain MT-9 of *Corynebacterium* for three other rodents. Rabbits were found to be immune. Guinea pigs were susceptible when injected intravenously. The white rat proved to be susceptible when inoculated intravenously or intraperitoneally. The intravenous LD_{50} for 100 g rats was approximately 4 million viable bacilli. At this dose, those animals which died showed multiple abscesses of the lungs. The pus in these lesions contained the bacilli in large numbers. Many animals also showed abscesses in the kidneys and abscesses in the liver as illustrated in figure 1. Polyarthritis occurred in all the survivors of the intravenous LD_{50}. The lesions in the liver and kidneys appeared during the first week after inoculation and were generally not seen in animals autopsied two or more weeks following infection. The abscesses in the lungs persisted for some time and have been observed in animals autopsied two months after infection. They first became evident about three days after inoculation as small white dots about two mm in diameter, distributed quite uniformly throughout the lungs. The adjacent tissue was generally not inflamed. The size of the abscesses increased with time, consolidation occurred, and lesions often developed with a diameter of 10 mm. Rats autopsied 4 months following infection showed scar tissue in the lungs where abscesses had apparently healed. All rats used were of the Sprague-Dawley strain.

FIG. 1.—Internal organs of white rat 6 days after intravenous inoculation of approximately 1,600,000 viable bacilli as determined by plate count. Abscesses in liver and lungs produced by strain MT-9 of *Corynebacterium* may be readily observed.

The polyarthritis first appeared two days following infection, and the joint swellings generally increased to reach a maximum three weeks later. The tarsus was most frequently involved, but swellings also occurred in the carpus and joints of the digits as illustrated in figure 2. In some cases the arthritis subsided, especially where animals had been infected with small doses. In

others, the arthritis persisted and gradually became worse. Some joints showed calcification and ankylosis. The diphtheroid bacillus was isolated in pure culture from such lesions 5 months after the inoculation. The incidence of arthritis at various doses is illustrated in table 1.

Histologic studies

Histopathological studies were carried out on 48 rats and 11 mice that died or were killed following inoculation with the MT-9 strain of *Corynebacterium*. The results of these studies are discussed below.

Multiple abscesses in the kidneys were observed in 29 out of 36 rats (81%) and in 10 out of 10 mice (100%). Some of the kidney lesions consisted of zones of necrosis surrounded by lymphocytes, neutrophiles, and monocytes, with clumps of bacteria scattered about the lesions as illustrated in figure 3.

TABLE 1.—*Mortality and incidence of arthritis after intravenous inoculation of 100 white rats with MT-9 strain of* Corynebacterium.

Inoculum based on plate count	No. of animals used	Days after inoculation	No. dead	No. of survivors without arthritis	No. of survivors with arthritis	% of survivors with arthritis
78,000,000	20	7	15	0	5	100
		14	20			
4,000,000	20	7	5	3	12	80
		14	9	0	11	100
		21	9	0	11	100
		28	9	0	11	100
1,600,000	20	7	3	6	11	65
		14	4	3	13	81
		21	5	6	9	60
		28	5	6	9	60
800,000	20	7	2	11	7	39
		14	4	7	9	56
		21	4	6	10	62
		28	4	4	12	75
200,000	20	7	0	16	4	20
		14	0	9	11	55
		21	1	9	10	53
		28	1	9	10	53
65,000	20	7	1	17	2	11
		14	4	12	4	25
		21	4	14	2	13
		28	4	11	5	31

Lesions in the lungs were seen in 41 out of 45 rats (91%) and in 5 out of 9

FIG. 2.—White rat 24 days after intravenous inoculation of approximately 4,000,000 viable bacilli as determined by plate count. Arthritis of the tarsus, carpus, and joints of the digits produced by strain MT-9 of *Corynebacterium* may be readily observed.

mice (56%). These lesions contained clumps of bacteria and sometimes involved most of the lobe. There was an infiltration of lymphocytes, neutrophiles, and monocytes. In a few rat lungs some of the alveoli were distended with serous fluid. In many of the lungs there was a peribronchial infiltration of monocytes.

Heart lesions were seen in 13 out of 41 rats (32%) and in 2 out of 7 mice (29%). The heart involvement in both species consisted of scattered areas of necrosis in the myocardium with an infiltration of lymphocytes and neutrophiles. The older lesions showed an increase in fibrous tissue around the abscesses with clumps of bacteria scattered throughout as seen in figure 4.

Liver lesions occurred in 21 out of 38 rats (55%) and in 2 out of 7 mice (29%). These consisted of scattered abscesses around the central veins.

No lesions were observed in the spleen, adrenals, and lymph nodes of the mice, but in the rats small areas of focal necrosis and some edema were seen in these organs.

One mouse brain showed gross lesions and was examined microscopically. Multiple abscesses containing clumps of bacteria were seen in the cerebrum and the cerebellum.

Thirty-three arthritic joints of rats and three arthritic joints of mice were examined. In a typical section from the rat taken 10 days after inoculation, the muscle and soft tissue around the joint showed marked edema. There was thickening and edema of the joint capsule with capillary proliferation and abundant infiltration of mononuclears and neutrophiles. The synovial membrane showed thickening and proliferation, and the joint capsule was filled with a fibrino-purulent exudate. The nearby bone showed the periosteum lifted up by the proliferation of osteo-

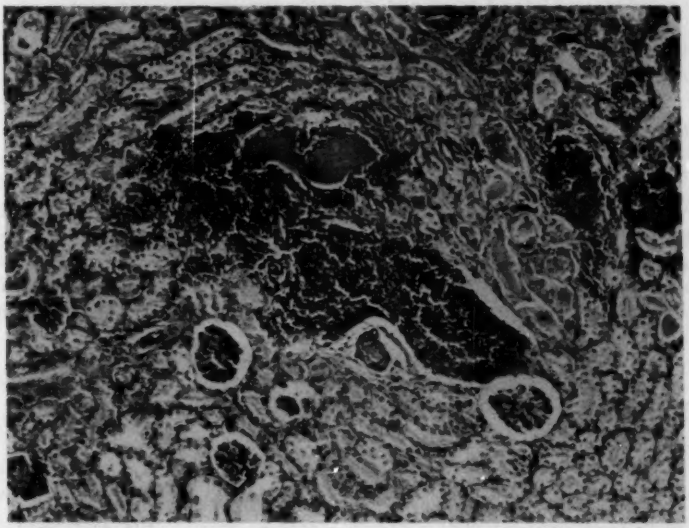

FIG. 3.—Kidney of rat 7 days after inoculation with strain MT-9 of *Corynebacterium*. Multiple abscesses and clumps of bacteria in the cortex are shown.

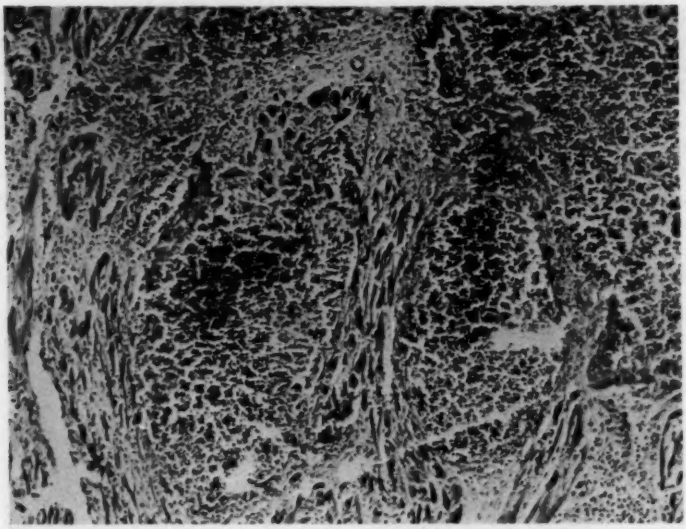

FIG. 4.—Heart of mouse 6 days after inoculation with strain MT-9 of *Corynebacterium*. Multiple abscesses with clumps of bacteria in the myocardium are shown.

FIG. 5.—Elbow joint of rat 10 days after inoculation with strain MT-9 of *Corynebacterium*. Partial destruction of epiphyseal line and of the humerus are shown. Fibroblastic proliferation of the endosteum and periosteum is also shown.

blasts and the periosteum and endosteal surfaces of the bone showed erosion. The medullary cavity showed marked fibrosis, and the epiphyseal line was partially destroyed. The articular surfaces of the joint showed partial replacement by endosteal fibroblastic proliferation, and clumps of bacteria were seen in the lesions as illustrated in figure 5.

In later stages of the infection there was marked thickening of the joint capsule and partial collapse of the articular surfaces with bits of bone and cartilage lying in the joint cavity.

In one of the three arthritic joints from mice there were three small abscesses of the soft tissue containing clumps of bacteria, and the abscesses were infiltrated with monocytes and neutrophiles. The joint capsule was edematous, showed some capillary proliferation, and was infiltrated with monocytes. The articular cartilages and the bone shaft were eroded at several points.

Relationship of our strain to those previously described

The question of the relationship of strain MT-9 of *Corynebacterium* to those previously described now arose. A search through the literature on the subject revealed that the first related species was described by Kutscher (1894). This author reported the isolation of a diphtheroid bacillus from a caseous abscess in the lung of a mouse which had died spontaneously. He named the organism *Bacillus pseudotuberculosis murium* because of the gross resemblance of the lesions in mice to those produced by *Mycobacterium tuberculosis* in other susceptible animals. Intraperitoneal inoculation of this bacillus into mice produced characteristic lesions in the kidneys, spleen, and liver. Mice could be infected by intrathoracic injection and by inhalation of dried bacilli but could not be infected through the intestinal tract. Guinea pigs, rabbits, cats, and dogs were immune to all methods of infection with these organisms.

Next, Bongert (1901) described a highly contagious disease of mice produced by injection of material from horses dying of pneumonia. The infected mice showed caseous nodules in the lung, liver, spleen, kidneys, and heart. The responsible organism was found to be a diphtheroid bacillus which the author felt was not the same species as that previously described by Kutscher. Bongert's bacillus produced a heat labile toxin which killed mice when administered by injection or feeding. Mice given subcutaneous injections of the bacilli frequently died with no sign of involvement of the internal organs. Rats, guinea pigs, rabbits, doves, and hens were completely immune to subcutaneous, intraperitoneal, and intravenous injections of large doses of live bacilli. Bongert named his strain *Corynethrix pseudotuberculosis murium*.

The Medical Research Council of Great Britain prepared a monograph on diphtheria (1923) edited by Andrewes et al. The authors state that the *Bacillus pseudotuberculosis murium* of Kutscher is probable identical with the *Corynethrix pseudotuberculosis murium* of Bongert. They also mention fermentation studies done on two similar organisms isolated by Professors Topley and Browning and assumed to be identical with the strain described by Kutscher. They felt that the organism was indistinguishable morphologically from *Corynebacterium diphtheriae*.

Holzhausen (1927) isolated a diphtheroid from mice which had been inoculated with brain emulsion from a rabid dog. The organism produced an overwhelming infection in mice and was taken up by the endothelium of the

blood vessels. White and gray mice were found to be highly susceptible. They could be infected by scratching the ear with a contaminated needle. The organism was hemolytic and produced no toxin. It was named *Corynebacterium murisepticum*.

Condrea (1930) described an extremely contagious benign disease of white mice characterized by the appearance of small subcutaneous nodules in the dorsal region, occasionally on the flanks. A diphtheroid bacillus was isolated from these lesions and named *Corynebacterium muris*. A prick with an infected needle reproduced the disease in all its characteristics. Intravenous inoculation of this culture into mice killed the animals in from one to 10 days, depending on the dose. Those animals which died 10 days after infection showed numerous abscesses in the kidneys and lungs. The organism was found to be avirulent for the rabbit.

Fischl, Koech and Kussat (1931) isolated a diphtheroid bacillus from the knee joint of a white mouse with a swollen leg. The organism was described as being highly pathogenic for mice and rats by intra-articular injection into the knee joint, but the infection was localized at the site of inoculation. Intraperitoneal injection produced systemic toxic effects without visible tissue damage. Subcutaneous and intraocular injections also produced only localized lesions. The authors named the organism *Corynebacterium arthritidis muris*.

Kutscher, Bongert, Holzhausen, and Condrea made no mention of the production of arthritis by the organisms they described. The bacillus of Fischl, Koech, and Kussat produced arthritis only by injection directly into the joint and produced no observable lesions when injected intraperitoneally. Intravenous administration was not described.

These 5 papers were the only ones found having a direct bearing on the identity of strain MT-9 except for a report by Reed (1902) which was considered unreliable and which contributed nothing that had not previously been reported by Kutscher and Bongert. From the description of the organisms given by the authors it is evident that marked differences in cultural characteristics and animal pathogenicity were observed. From comparisons of the information obtained from these reports, we have concluded that the organisms described by Kutscher, Condrea, and ourselves, are very similar, but probably not identical. Kutscher's strain produced characteristic colonies in 24 hours which were light yellow in color and finely granular at the center. MT-9 produces characteristic colonies only after 48 hours of incubation even on enriched mediums. These are white and have a smooth glistening surface. Kutscher carried out so few biochemical tests that adequate comparison is not possible. Condrea's organism produced acid in broth containing arabinose, dextrine, inulin, and sorbitol, whereas MT-9 does not ferment these sugars.

The bacilli described by Bongert, Holzhausen, and Fischl, Keoch, and Kussat appear to have been separate species which have not been reported since by other workers. They differ with each other and with the organisms described by Kutscher, Condrea, and ourselves. It seems probable, therefore, that there is in nature a group of related species of the genus *Corynebacterium*, showing various degrees of pathogenicity for mice and rats. Because of the state of the literature on the subject and the lack of the original cultures, no other conclusions regarding species identification can be made at the present time.

SUMMARY

An organism resembling *Corynebac-*

terium kutscheri has been isolated from a subcutaneous abscess and from throat cultures of white mice and also from the pus in lung abscesses of uninoculated white rats. The cultural characteristics and biochemical reactions of the organism are presented. The experimental intravenous and intraperitoneal infection of white mice with this bacillus produces a disease similar to that described by Kutscher and Condrea. The organism is also pathogenic for white rats when injected intravenously or intraperitoneally. By the former route a disease is produced with polyarthritis as one of the prominent features. The experimental infection of white rats with similar organisms has not previously been described.

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EXPERIMENTAL ARTHRITIS IN ALBINO RATS AND MICE PRODUCED BY ALPHA TYPE STREPTOCOCCI

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In a search for bacteria from human sources which would produce experimental arthritis in albino rats and mice, throat cultures were obtained from 5 adults. These cultures were inoculated intravenously into white mice, and the animals were observed closely thereafter. The inoculum consisted of a broth suspension of the 24-hour growth of the specimen streaked on Loeffler slants containing .01% of potassium tellurite. Forty-eight hours after the injection all 5 mice inoculated with the cultures from one of the subjects showed arthritis in one or more leg joints. The animals inoculated with throat cultures from the 4 other subjects showed no ill effects. From the swollen joints of the mice with arthritis we obtained a pure culture of a strain of alpha type *Streptococcus*. All white mice used were of the National Institutes of Health general purpose strain.

Description of the first strain: JST

This organism, designated JST, is Gram positive with spherical or ellipsoidal cells approximately .6 μ in diameter, usually in short chains. On blood agar after 24 hours of incubation at 37 C, the colonies are one mm in diameter. They are round, white, convex, opaque, with smooth margin and glistening surface. The colonies are surrounded by an alpha type zone when grown on the surface or in the depth of the plate. After the same incubation period on nutrient agar, the colonies are one-half mm in diameter. In nutrient broth the organism produces uniform turbidity and a moderate amount of sediment. Gelatin is not liquefied. Litmus milk is acidified and coagulated. There is no visible growth on potato. Arginine and sodium hippurate are hydrolyzed, but esculin is not. Catalase is not produced. Ammonia is not produced from peptone. Nitrate is reduced to nitrite. Indol and hydrogen sulfide are not formed. The organism is aerobic, facultatively anaerobic. Acid but no gas is produced in broth containing dextrine, galactose, glucose, lactose, levulose, maltose, mannose, salicin, starch, sucrose, and trehalose. No acid is formed from adonitol, amygdaline, arabinose, cellobiose, dulcitol, erythritol, glycerol, inositol, inulin, mannitol, raffinose, rhamnose, sorbitol, or xylose. The organism tolerates 4% of sodium chloride but is inhibited by .01% of methylene blue.

The experimental infection in mice

The organism is pathogenic for white mice when injected intravenously, intraperitoneally, or subcutaneously. The intravenous LD_{50} for 20 g mice is approximately 5 million organisms as determined by plate count. At this dose the animals which die show multiple abscesses of the kidneys, a high incidence of abscesses in the heart muscle, and a high incidence of arthritis. The carpus and tarsus are most frequently involved, but arthritis of knee, hip, elbow, and joints of the digits occurs frequently. The organism may be isolated in pure culture from these joints during the first few weeks of the infection. Figure 1 illustrates arthritis of the tarsus in a white mouse produced by strain JST.

The pus in abscesses produced by this organism has a characteristic orange color. The arthritis of the tarsus and carpus is often observable 18 hours following the injection. The swelling generally reaches a maximum in one week and gradually subsides thereafter. Arthritis of the hip joint is generally not

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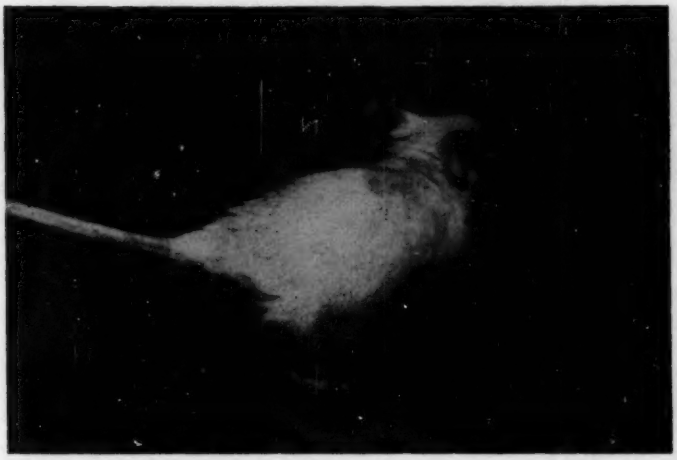

FIG. 1.—White mouse 28 days after intravenous inoculation of approximately 10,000,000 viable organisms as determined by plate count. Arthritis of tarsus, produced by strain JST of alpha type *Streptococcus*, is readily observed.

observable until several weeks after inoculation but persists longer than the swellings of most of the other joints. The injury to some of the joints is apparently permanent. Table 1 illustrates the mortality and incidence of arthritis in mice at various doses of strain JST.

The organism is pathogenic for white rats. The intravenous LD_{50} for 100 g rats is approximately 20 million viable

FIG. 2.—White rat 8 days after intravenous inoculation of approximately 15,000,000 viable organisms as determined by plate count. Arthritis of the carpus, produced by strain JST of alpha type *Streptococcus*, is readily observed.

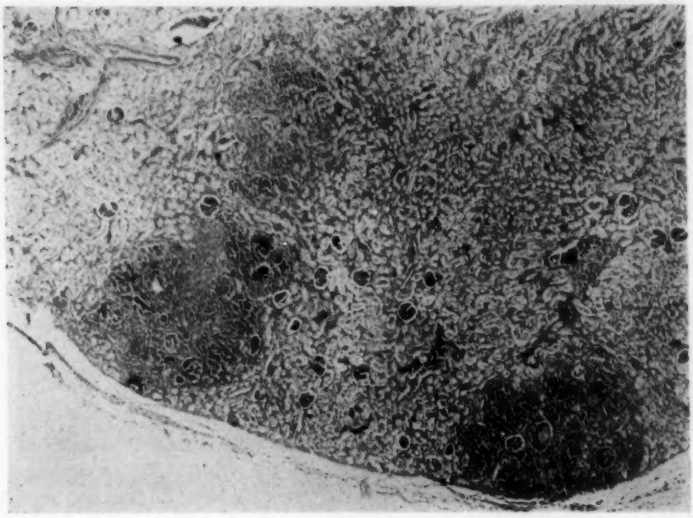

FIG. 3.—Kidney of rat showing multiple abscesses in the cortex and medulla with clumps of bacteria in the lesions and glomeruli.

organisms as determined by plate count. At this dose those animals which die show the same type and distribution of lesions as was described for the white mouse. Figure 2 illustrates arthritis of the carpus in a white rat. All rats used were of the Sprague-Dawley strain.

Table 2 illustrates the mortality and

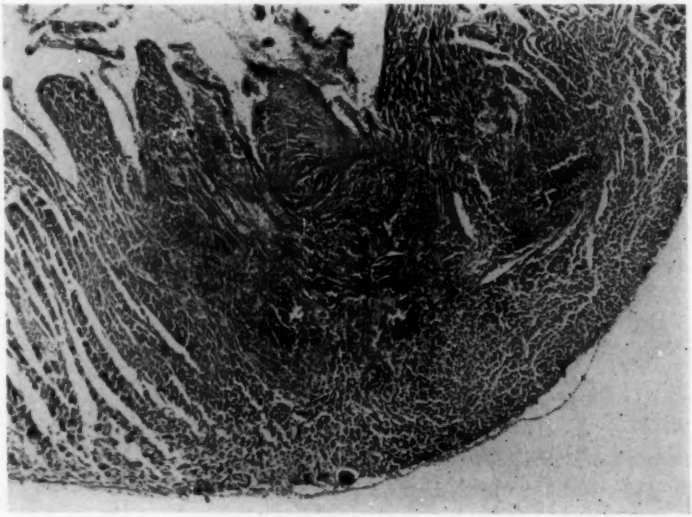

FIG. 4.—Heart of rat showing multiple abscesses in the myocardium and endocardium.

TABLE 1.—*Mortality and incidence of arthritis after intravenous inoculation of 20 g white mice with JST strain of alpha type* Streptococcus.

Inoculum base on plate count	No. of animals used	Days after inoculation	No. dead	No. of survivors without arthritis	No. of survivors with arthritis	% of survivors with arthritis
9,000,000	20	2	0	10	10	50
		7	4	3	13	81
		14	9	4	7	64
		22	9	2	9	82
		29	11	2	7	78
6,000,000	20	2	1	5	14	74
		7	5	3	12	80
		14	14	3	3	50
		21	15	3	2	60
		28	15	3	2	60
3,500,000	20	2	0	9	11	55
		7	0	8	12	60
		16	1	11	8	42
		21	2	9	9	50
		28	2	11	7	41
1,200,000	20	2	0	15	5	25
		7	0	10	10	50
		14	0	10	10	50
		21	0	9	11	55
		28	0	8	12	60

TABLE 2.—*Mortality and incidence of arthritis after intravenous inoculation of 100 g white rats with JST strain of alpha type* Streptococcus.

Inoculum based on plate count	No. of animals used	Days after inoculation	No. dead	No. of survivors without arthritis	No. of survivors with arthritis	% of survivors with arthritis
220,000,000	10	2	0	0	10	100
		15	10			
22,000,000	10	2	0	0	10	100
		15	4	1	5	83
		23	4	1	5	83
		56	4	4	2	33
15,000,000	12	2	0	3	9	75
		10	2	3	7	70
		23	3	3	6	67
		43	3	5	4	44
7,500,000	11	2	0	4	7	64
		10	2	6	3	33
		23	2	6	3	33
		43	2	6	3	33
3,750,000	11	2	0	8	3	27
		10	0	6	5	45
		23	1	7	3	30
		43	2	6	3	33
2,200,000	10	2	0	6	4	40
		15	2	7	1	13
		23	2	7	1	13
		56	2	7	1	13

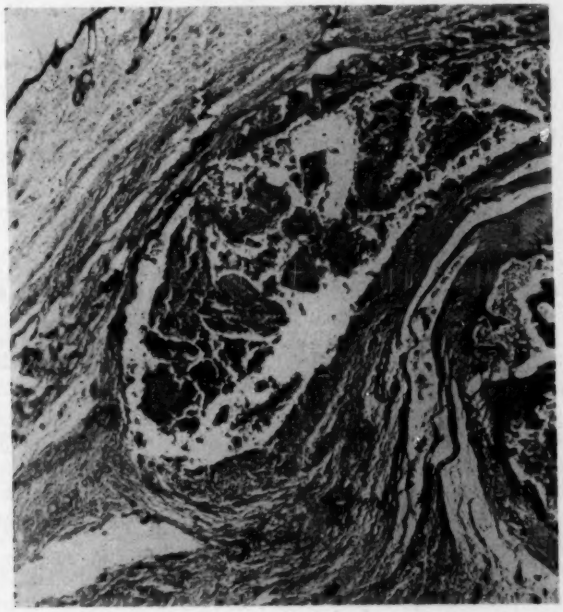

FIG. 5.—Knee joint of rat, 6 days after inoculation, showing edema, proliferation of blood capillaries, and a large abscess of soft tissue of the joint.

incidence of arthritis in white rats at various doses of strain JST.

Tissue from 20 rats and 20 mice which died of streptococcus infection were submitted for histopathological studies. Most of these animals had received an inoculum of approximately one LD_{50}.

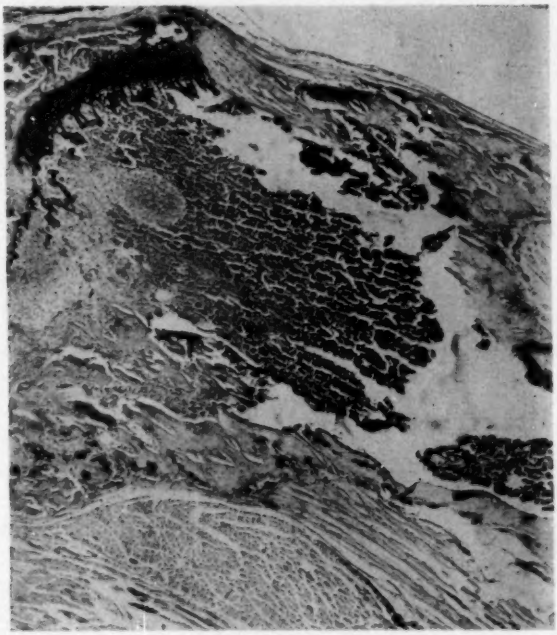

FIG. 6.—Knee joint of mouse, 15 days after inoculation, showing multiple abscesses of joint capsule and of medullary cavity of the tibia. Several clumps of bacteria can be seen in the necrotic area at the lower end of the epiphyseal line.

The incidence and type of lesions observed in the organs studied are listed below.

Kidney lesions were found in 10 of 16 rats (62.5%), and in 18 of 20 mice (90%). Microscopically the kidney sections in both animals showed multiple abscesses in the cortex and medulla with clumps of streptococci in the abscesses and in the glomeruli (fig. 3).

Heart lesions were found in 12 of 16 rats (75%) and in 8 of 20 mice (40%). The lesions of the heart from both species consisted of multiple abscesses containing streptococci in the myocardium and endocardium. The lesions most frequently seen were located in the myocardium at the apex of the heart (fig. 4).

Liver lesions were seen in 4 of 14 rats (28.5%), and in 9 of 18 mice (50%). The liver lesions in the rats consisted of small areas of focal necrosis, and occasionally a small abscess was seen. In the mice the lesions consisted of small abscesses around the central veins.

Lesions in the lungs were observed in 2 of 17 rats (11.7%) and in 1 of 15 mice (6.6%).

Other organs in the mice showed no lesions, but in the rat two spleens and one adrenal showed a few small areas of focal necrosis. No cases of acute monocular purulent endophthalmitis as re-

ported by Rothbard (1940) were seen.

Thirty-six arthritic joints of rats and 30 arthritic joints of mice were sectioned and examined microscopically. In the early stage of the infection the soft tissues of the joint are edematous and show distention and prominence of the capillaries. There is an abundant infiltration of neutrophils and lymphocytes in focal areas, and scattered clumps of streptococci are present in the lesions. In the later stages these infiltrated areas coalesce to form large abscesses that become encapsulated with a dense fibrous connective tissue capsule (fig. 5). In some animals lesions are seen in the bones and tissues of the joints, and in these cases there is a proliferation and thickening of the synovial villae, proliferation of the periosteum and endosteum, and abscess formation in the medullary cavity, epiphysis, and articular surfaces (fig. 6). In the chronic stage there is a replacement of the necrotic tissue by cartilage and bone forming elements and deposition of calcium.

Isolation of the second strain of Streptococcus: *FJH*

A second strain of alpha type *Streptococcus*, designated FJH, was then isolated from the swollen leg joint of a mouse inoculated intravenously with cultures from the throat of another human subject who had given 8 negative samples previously. Strain FJH is similar to JST morphologically and in all but two biochemical reactions. Strain JST ferments lactose and produces acid and coagulation in litmus milk. Strain FJH does not ferment lactose and causes no change in litmus milk. The two cultures are different serologically. An antiserum prepared by immunizing rabbits with JST agglutinates that organism to to a titer of 1:5120. The same antiserum has a titer of 1:320 against strain FJH. An antiserum prepared by immunizing rabbits with strain FJH agglutinates that organism to a titer of 1:2560. It agglutinates strain JST to a titer of 1:320. The two organisms also differ in virulence for rats and mice. Strain FJH kills only three out of 20 white mice at an intravenous dose of 200 million viable organisms. The same dose of strain JST kills all the mice so exposed. The appearance and distribution of joint lesions are similar for both strains. However, the gross abscesses in the heart muscle produced by strain JST are generally not caused by strain FJH. The second strain is of lower virulence for both rats and mice and produces lesions mainly in the joints and kidneys. Table 3 shows the mortality and incidence of arthritis after intravenous inoculation of white mice with this organism.

TABLE 3.—*Mortality and incidence of arthritis after intravenous inoculation of 20 g white mice with FJH strain of alpha type* Streptococcus.

Inoculum based on plate count	No. of animals used	Days after inoculation	No. dead	No. of survivors without arthritis	No. of survivors with arthritis	% of survivors with arthritis
200,000,000	20	2	0	3	17	85
		7	0	7	13	65
		14	3	5	12	71
		21	3	8	9	53
		28	3	10	7	41

DISCUSSION

The two strains of alpha type streptococci just described do not correspond exactly with any of the alpha type streptococci listed in Bergey's Manual. They resemble *Streptococcus mitis* most closely in biochemical reactions. However, our strains hydrolyze arginine and sodium hippurate, whereas *Str. mitis* does not. Strains JST and FJH tolerate 4% sodium chloride. *Str. mitis* tolerates 2% but not 4% of sodium chloride. Our organisms produce uniform turbidity in broth supernatant, *Str. mitis* produces a clear supernatant or granular turbidity.

We have searched the literature and found no report of the production of polyarthritis in rats and mice by intravenous injection of alpha type streptococci. Experimental arthritis in rabbits has been studied for many years and may be produced by a variety of bacteria. Cecil, Angevine, and Rothbard (1939) reported joint involvement in rabbits following intravenous injection of alpha type streptococci, beta type streptococci, *Staphylococcus aureus*, *Diplococcus pneumoniae*, and *Salmonella*

schottmuelleri. Rothbard (1940) reported production of arthritis in albino rats by intravenous inoculation of a Group A beta type *Streptococcus* isolated from the blood stream of a patient with septicemia. His culture was of lower virulence for his rats than JST strain is for ours and of higher virulence respectively than the FJH strain. The distribution and nature of the joint involvement was quite similar to the results we have obtained with our strains. Rothbard does not mention the effect of his organism on mice. Experimental polyarthritis may also be produced in mice by injection of *Streptobacillus moniliformis* as reported by Levaditi et al (1932) and in rats by the injection of pleuropneumonia-like organisms as reported by Findlay et al (1939). Several other animals are also susceptible to joint involvement following intravenous injection of suitable bacteria. Ward (1922) reported production of polyarthritis in swine by intravenous injection of cultures of *Erisipelothrix rhusiopathiae*. Collins and Goldie (1940) reported production of polyarthritis in swine, sheep, and horses by intravenous injection of the same organism. Marsh (1933) reported similar results in lambs. The same author (1947) recently reported isolation of *Corynebacterium ovis* from naturally occurring arthritis of lambs. However, we were unable to find any reports on production of polyarthritis in rats and mice by alpha type streptococci. Either our strains have not been isolated before or, if isolated, have not been tested by intravenous inoculation into susceptible rats and mice.

We have succeeded in isolating alpha type streptococci, which produce a high incidence of polyarthritis in white mice and rats, from throat cultures of two out of 5 human subjects tested. It seems probable, therefore, that similar organisms are widely distributed. No doubt, they belong to a group showing various degrees of pathogenicity for rats and mice. On the basis of the characteristics of the two strains we have studied, we would expect the group to be serologically heterogeneous, but quite similar culturally. The group may be useful for studies in the fields of experimental arthritis, chemotherapy, antibiotics, and endocrinology.

SUMMARY

Two strains of alpha type *Streptococcus* have been isolated culturally which produce a high incidence of polyarthritis in albino rats and mice when injected intravenously in suitable amounts. The cultural characteristics and biochemical reactions of the organisms are given. The disease produced in albino rats and mice with these strains is described.

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